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WITH EXTENDED CIRCULATORY LONGEVITY

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# **Generation of Recombinant Human AChE OP- Scavengers with Extended Circulatory Longevity**

## **MIDTERM REPORT**

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## CONTENTS

	<u>Page</u>
I. GENERAL INTRODUCTION	8
II. THE EFFECT OF POLYETHYLENE GLYCOL CONJUGATION ON THE CIRCULATORY LIFE-TIME OF ACETYLCHOLINESTERASE MOLECULAR FORMS DIFFERING IN THEIR POST-TRANSLATIONAL PROCESSING	12
Introduction	12
Methods	14
- PEG-conjugation reaction and analysis of the products	14
- Enzyme activity	14
- Pharmacokinetics in mice	14
- Sucrose gradient	15
- Non-denaturing deglycosylation of AChE	15
- MALDI-TOF Analysis of Basic Glycan Structures	15
- Release, recovery, purification and labeling of N-glycans	15
- Sialic acid removal	15
- Mass spectrometry	15
Results and Discussion	17
- Effect of enzyme subunit assembly on the circulatory retention of PEGylated rHuAChE	17
- Effect of glycan quantity and composition on the circulatory retention of PEGylated rHuAChE	18
- Effect of lysine content on the circulatory retention of PEGylated rHuAChE	25
III. GENERATION OF PEG-CONJUGATED rHuAChE DEVOID OF MAMMALIAN POST-TRANSLATION MODIFICATION - BACTERIALLY EXPRESSED SYNTHETIC rHUACHE GENE	29
Introduction	29
Methods	32
- Planning and construction of a synthetic human AChE gene	32
- <i>In-vitro</i> expression system	32
Results	33
- Construction of a Human AChE Synthetic Gene	33
- Characterization of the Synthetic Human AChE Gene Product in an <i>In-vitro</i> Transcription-Translation System	38

CONTENTS (cont'd)

	<u>Page</u>
IV. REGULATION OF ACETYLCHOLINESTERASE CIRCULATORY RESIDENCE BY POST-TRANSLATIONAL PROCESSING AND SPECIES-SPECIFIC AMINO ACID EPITOPES	39
Introduction	39
Methods	40
- Cell culture techniques, enzyme production and purification of rAChEs	40
- DNA sequencing	40
- Generation of the RhAChE cDNA	40
- Enzyme activity	41
- Removal of sialic acid from rAChE	41
- <i>In-vitro</i> tetramerization of rAChE	41
- MALDI-TOF analysis of glycans	41
- Conjugation of polyethylene glycol to AChE	42
- Animal procedures	42
- Clearance experiments and analysis of pharmacokinetic profiles in mice and rhesus macaques	43
- Clearance experiments of native and radiolabeled RhBChE in rhesus macaques	44
Results and Discussion	45
- Pharmacokinetics of recombinant BoAChE forms obey the same hierarchical rules in rhesus macaques and mice	45
- The circulatory elimination patterns of recombinant human AChEs in rhesus macaques appear to deviate from the classical hierarchical rules established in mice	47
- Cloning, expression and N-glycan analysis of various posttranslationally modified recombinant RhAChE forms	49
- Pharmacokinetic profiles of recombinant RhAChE in Rhesus macaques and mice	53
- Facilitated elimination of cholinesterases from the circulation of rhesus macaques is restricted to primate AChE but not to primate BChE	57
- A possible role for amino acid domains in the differential pharmacokinetic behavior of bovine and primate AChEs in rhesus macaques	58
- The effect of masking of primate-specific amino-acid domains by PEGylation on the circulatory life-time of AChE in rhesus macaques	60
V. KEY RESEARCH ACCOMPLISHMENTS	64
VI. REPORTABLE OUTCOME	66
VII. CONCLUSIONS	67
VII. REFERENCES	69

LIST OF FIGURES

	<u>Page</u>
1- Sucrose-gradient sedimentation profiles of FBS-AChE and $\Delta$ C-rHuAChE	17
2- Pharmacokinetic profiles of non-modified and PEG-modified FBS-AChE and PEG-modified $\Delta$ C-rHuAChE	18
3- Pharmacokinetic profiles of non-modified and PEG-modified monoglycosylated N350Q/N464Q rHuAChE and PEG-modified $\Delta$ C-rHuAChE	19
4- The basic structures and relative abundances of desialylated N-glycans released from monoglycosylated N350Q/N464Q rHuAChE and from wild-type rHuAChE.	21
5- Pharmacokinetic profiles of PEG-modified $\Delta$ C-rHuAChE containing different levels of sialic acid	22
6- SDS-PAGE analysis of $\Delta$ C-HuAChE before and after non-denaturing N-glycan treatment	23
7- Pharmacokinetic profiles of non-modified and PEG-modified $\Delta$ C-rHuAChE devoid of N-glycans	24
8- Schematic representation of the distribution of lysine residues along the HuAChE sequence and the C-terminal sequence of the C580A and $\Delta$ C-rHuAChE forms	26
9- Pharmacokinetic profiles of non-modified and PEG-modified C580A rHuAChE and PEG-modified $\Delta$ C-rHuAChE.	27
10- Representative codon usage and GC content of native rHuAChE, synthetic HuAChE and several microorganisms	36
11- Schematic presentation of the assembly of the synthetic HuAChE gene.	37
12- Description of the assembled synthetic AChE gene and characterization of its protein product in an <i>in-vitro</i> transcription/translation system.	38
13- Comparison of the circulatory elimination profiles of various molecular forms of rBoAChE and rHuAChE in rhesus macaques.	46
14- Determination of rhesus AChE gene sequence	50
15- Characterization of recombinant rhesus AChE	52
16- Circulatory elimination profiles of various molecular forms of rRhAChE in mice and in rhesus macaques	54
17- Effect of sialylation, oligomerization and glycan loading on the circulatory residence of bovine, human and rhesus AChEs in mice or rhesus macaques	56

	<u>Page</u>
18- Circulatory elimination profiles of labeled and non-labeled rhesus butyrylcholinesterase (BChE) in rhesus macaques	58
19- Mapping of divergent amino acids between BoAChE and RhAChE/HuAChE onto the three-dimensional model of HuAChE	61
20- Pharmacokinetic profiles of PEGylated rRhAChE and PEGylated rHuAChE in rhesus macaques	63

#### LIST OF TABLES

1- Pharmacokinetic parameters of non-modified and PEG-modified FBS-AChE and PEG-modified $\Delta$ C-rHuAChE.	18
2- Pharmacokinetic parameters of non-modified and PEG-modified monoglycosylated N350Q/N464Q rHuAChE and PEG-modified $\Delta$ C-rHuAChE	20
3- Pharmacokinetic parameters of non-modified and PEG-modified C580A rHuAChE and PEG-modified $\Delta$ C-rHuAChE	27
4- Comparison of the codon usage of native rHuAChE to that of several micro-organisms and the synthetic HuAChE	34
5- Pharmacokinetic parameters of rBoAChE and rHuAChE in rhesus macaques	47
6- Pharmacokinetic parameters of rRhAChE in rhesus macaques and mice	53

## I. GENERAL INTRODUCTION

The primary role of acetylcholinesterase (acetylcholine acetylhydrolase 3.1.1.7, AChE) is the termination of impulse transmission in cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (ACh). The enzyme has been the subject of intense research due to its focal position in several fields of interest. Modification in the levels of human brain AChE have been reported in various disorders such as Down's syndrome (Yates *et al.*, 1980) and Alzheimer's disease (Coyle *et al.*, 1983). Several cholinesterase inhibitors have proven to be of value as medicinal agents and are used for the treatment of glaucoma or myasthenia gravis (Taylor, 1990). Some organophosphorus (OP) inhibitors of ChEs such as malathion and diazinon, act as efficient insecticides and have been widely used in combating medfly and other agricultural pests. Other OP compounds, such as the nerve agent sarin and soman, inhibit AChE irreversibly by rapid phosphorylation of the serine residue in the enzyme active site. The acute toxicity of these nerve agents is elicited in motor and respiratory failure following inhibition of AChE in the peripheral and central nervous system.

The current treatment regimes against nerve agent exposure are designed to protect life but are not able to prevent severe incapacitation. The high reactivity of ChEs towards OP-agents led to propose these biomolecules as exogenous scavengers for sequestration of toxic OP-agents before they reach their physiological target (Wolfe *et al.*, 1987; Raveh *et al.*, 1989; Broomfield *et al.*, 1991; Doctor *et al.*, 1992). The AChEs react irreversibly and on a molar basis, with the OP agents and therefore, the amounts of AChE required for treatment are high. This limitation could be overcome provided that the OP-enzyme conjugates could be efficiently reactivated before the excess OP has reached its physiological target. This goal is difficult to attain especially in cases where the OP-AChE conjugates undergo catalytic post-inhibitory processes termed aging. In native AChEs the spontaneous reactivation, through displacement of the phosphyl moiety from the active site, is usually very slow and unable to compete with the aging process, yet efficient enzyme reactivation can be achieved by various oxime nucleophiles. Our goal is therefore to generate enzymes based on the HuAChE template, the OP-adducts of which are more readily reactivated and are resistant to aging, yet still retain their high reactivity towards the OP agents. To meet this challenge, we as well as others initiated studies designed for better understanding of the functional architecture of the AChE active center. These include X-ray crystallography (Sussman *et al.*, 1991; Harel *et al.*, 1996; Raves *et al.*, 1997; Kryger *et al.*, 1998; Millard *et al.*, 1999; Bourne *et al.*, 1999; Kryger *et al.*, 2000); site directed mutagenesis and molecular modeling together with kinetic studies of the AChE muteins with substrates and reversible inhibitors (Ordentlich *et al.*, 1993; Barak *et al.*, 1994; Shafferman *et al.*, 1992; Vellom *et al.*, 1993; Radic *et al.*, 1992; Radic *et al.*, 1993; Shafferman *et al.*, 1995; Ordentlich *et al.*, 1996; Ariel *et al.*, 1998; Ordentlich *et al.*, 1998). The functional role of the various active center subsites identified this way include: a) the esteratic site containing the catalytic triad Ser203,



His447 and Glu334; b) the "oxyanion hole" consisting of residues Gly121, Gly122 and Ala204; c) the "anionic subsite" or the choline binding subsite -Trp86; d) the hydrophobic site for the alkoxy leaving group of the substrate containing an "aromatic patch" that includes residues Trp86, Tyr337 and Phe338; e) the acyl pocket - Phe295 and Phe297 and f) the peripheral anionic subsite (PAS) including residues Tyr72, Asp74, Tyr124, Trp286 and Tyr341. Recently, we have examined the specific involvement of all these elements of the active center functional architecture, in determining reactivity and specificity of human AChE (HuAChE) towards different OP-inhibitors. We have also demonstrated the ability to generate novel enzymes that are more efficient in OP scavenging through the combined effects of improved activity toward the OP-agents together with higher resistance to the aging process (Ordentlich *et al.*, 1996; Shafferman *et al.*, 1996). Such studies, by us and others (see review in Taylor and Radic, 1994; Hosea *et al.*, 1995; Millard *et al.*, 1995; Hosea *et al.*, 1996; Lockridge *et al.*, 1997), reveal the structural and mechanistic determinants of AChE activity that are essential for the design of true OP hydrolytic activity into the AChE mold. Information regarding the potential diversity of aging mechanisms is essential for further elucidation of the role of AChE active center in these processes, as part of the development of effective treatment for human intoxication by organophosphorus agents, like certain insecticides and nerve agents.

Exploitation of the bioscavenging potential of the recombinant bioengineered mutant derivatives of AChE depends on large-scale production systems (Fischer *et al.*, 1993; Kronman *et al.*, 1992). However pharmacokinetic studies (Kronman *et al.*, 1992; Mendelson *et al.*, 1998) have shown that recombinant enzymes generated by these systems, relying on either bacterial or mammalian cells, are retained in the circulation of experimental animals for much shorter periods of time than native fetal bovine serum acetylcholinesterase (FBS-AChE) or human serum butyrylcholinesterase (BChE). Therefore, deciphering the mechanisms involved in clearance of cholinesterases from the bloodstream is of importance for the development of enzyme-based bioscavengers for treatment of organophosphorus poisoning. Our previous studies (Kronman *et al.*, 1995) have shown that the structures of the appended glycans of rHuAChE, and in particular the distal termini of these glycan projections, constitute a major factor in determining the circulatory duration of rHuAChE. This was exemplified by establishing the major role of N-glycan sialylation in determining the circulatory lifetime of rHuAChE. As a continuation of these studies, we have demonstrated (Chitlaru *et al.*, 1998) that production of rHuAChE with highly sialylated glycans (achieved by genetic modification of rHuAChE producer cell lines to coexpress a sialyltransferase gene) resulted in the generation of high levels of rHuAChE which exhibited increased circulatory retention. However, the highly sialylated enzyme was still eliminated from the circulation more rapidly than native serum-derived cholinesterases, suggesting that factors other than glycan sialylation also play a role in determining the circulatory fate of AChE.

To allow a comprehensive appreciation of the structural basis for the circulatory residence of AChE, we conducted a series of studies (Kronman *et al.*, 2000; Chitlaru *et al.*, 2001,) in which the post-translation-related differences between serum-derived native bovine AChE and its recombinant HEK-293-expressed counterpart were determined by MALDI-TOF analyses of N-glycans and sucrose-gradient analyses of enzyme subunit assembly status. These studies, together with extensive pharmacokinetic profiling of various AChE forms in mice, allowed us to demonstrate that sialic acid occupancy and enzyme oligomerization determine both together the circulatory residence of bovine AChE in an hierarchical manner. Thus, molecular species in which both glycan sialylation and enzyme subunit assembly were optimized, are characterized by exceedingly long circulatory life-time values which are comparable to that exhibited by native serum derived BoAChE. Further studies displayed that the circulatory longevity of the human form of AChE in mice is regulated by the same set and pattern of rules (Chitlaru *et al.*, 2001).

Increasing the number of appended N-glycans to human AChE was also shown to contribute to the circulatory performance of the enzyme (Chitlaru *et al.*, 2002). Extensive pharmacokinetic analyses of an array of enzyme species differing in their state of sialylation, subunit assembly and number of appended N-glycans, demonstrated that the effect of N-glycan addition on the pharmacokinetics of AChE is fully manifested only in the case of efficiently sialylated tetrameric forms of the enzyme. Thus, N-glycan loading operates as a third component together with sialylation and tetramerization, within a hierarchical set of rules governing the circulatory lifetime of AChE in mice. Whether additional traits of the enzyme also play a part in determining its circulatory fate, and whether the same set of post-translation-related factors preside over the circulatory residence of AChE in other animal systems as well, remain to be resolved.

In parallel, studies carried out in our laboratory (Cohen *et al.*, 2001) allowed us to determine a set of conditions for the generation of chemically modified recombinant AChE exhibiting full preservation of its catalytic abilities, and which resides in the circulation of mice for unprecedented periods of time (Mean residence time = 2100 min.), surpassing that of native serum-derived enzymes such the fetal bovine serum AChE (FBS-AChE).

The finding that AChE circulatory residence may be extended both by either post-translation modification or by PEG-conjugation, raises a question concerning the interrelationship between the effect of PEGylation and the enzyme processing, with regard to the overall effect on the pharmacokinetic performance of the enzyme. This line of studies should have practical implications on determining the source for large-scale recombinant AChE production for its subsequent transformation into a long-lived therapeutic product by PEGylation. The finding that enzyme processing and PEG-conjugation operate in an additive manner, resulting in the generation of enzyme forms exhibiting higher levels of circulatory residence, would necessitate enzyme-processing optimization prior to its subjection to PEG-appendage. On the other hand, if PEG-conjugation will prove to be equally effective regardless of the post-translation

processing state of the AChE enzyme, cost-effective production systems (e.g. bacterial, or other microorganisms) may be utilized for the large-scale production of recombinant human AChE.

Although PEG-conjugation of AChE leads to the generation of a circulatory long-lived species, the practical use of PEGylated AChE as an OP-bioscavenger would require that the enzyme product exhibit a high degree of homogeneity. This may demand the removal of some of the lysine residues which serve as targets for PEGylation, to allow production of uniformly PEGylated enzyme. To this end, a series of studies aimed to determine the effect of lysine removal on enzyme functionality, pharmacokinetic performance and structural homogeneity following PEGylation should be performed.

The present report covers the progress in all these research areas. The following section (section II) focuses on the interrelationship between PEG-conjugation and the post-translation modifications of proteins with regard to the overall pharmacokinetic performance of the protein. Section III describes the progress made towards the synthesis of a human *ache* gene optimized for expression in various microorganisms-based systems. Section IV deals in depth with the regulation of AChE retention in the circulation by post-translational processing and species-specific amino acid epitopes. In this section, we also included for the sake of completeness, pertinent data regarding AChE circulatory residence in rhesus macaques which were derived from a series of experiments funded by sources other than the USAMRMC contract, since they may have a major impact on the present project. Each of these sections includes a brief background introduction, methods and a result and discussion subsection. A summary of the main findings of the research concludes this report (Section VII).

## II. THE EFFECT OF POLYETHYLENE GLYCOL CONJUGATION ON THE CIRCULATORY LIFETIME OF ACETYLCHOLINESTERASE MOLECULAR FORMS DIFFERING IN THEIR POST-TRANSLATIONAL PROCESSING

### INTRODUCTION

Studies carried out in our laboratory demonstrated that PEG modification of AChE can result in the generation of bioactive enzyme exhibiting an improved pharmacokinetic profile (Cohen *et al.*, 2001). Examination of a set of conditions for the attachment of PEG to AChE, allowed us to determine that under certain conditions, 4 PEG molecules may be appended to monomeric rHuAChE, with minimal effect on the catalytic activity of the enzyme or on the reactivity towards active center inhibitors (e.g. edrophonium and diisopropylfluorophosphate (DFP)), peripheral site ligands (e.g. propidium), and even towards the bulky snake-venom toxin fasciculin II. The PEGylated AChE demonstrated prolonged circulatory retention rates which were characterized by an MRT exceeding 2000 minutes. Examination of MRTs of an array of PEGylated AChEs which differed one from another by their degree of modification, as well as by the length of the appended PEG chains, suggested that the overall increase in MRT is directly dependent on the molecular size of the modified enzyme (Cohen *et al.*, 2001).

In our previous studies under Contract DAMD17-00-C-0021, we demonstrated that recombinant AChE can be converted into a long-term circulating protein by a two-step modification procedure which results in high-level glycan sialylation and enzyme subunit tetramerization. These findings suggest that receptor-mediated removal and limited protein size, which are respectively remedied by sialylation and tetramerization, indeed play a crucial role in determining the circulatory behavior of rAChE. However, the possible interrelationship between PEG-conjugation and the post-translation modifications of proteins with regard to the overall pharmacokinetic profile of the protein product has not been explored. It therefore remains to be clarified whether PEGylation of rAChE exhibiting sub-optimal post-translation modifications will give rise to an enzyme species exhibiting maximal circulatory residence, or whether efficient AChE post-translation processing is required for the full scale manifestation of the effect of PEGylation on circulatory residence. For instance, the quantitative and qualitative features of enzyme glycosylation may contribute to the overall circulatory residence of PEGylated proteins resulting in the increased retention of glycosylated, fully-sialylated PEGylated AChE, as compared to nonglycosylated or undersialylated forms of PEGylated enzyme. Elucidation of the interrelationship between the state of post-translation processing and AChE PEGylation, might lead to the design of long-lived PEGylated-AChE, which benefits from the combined effect of

post-translation modifications with chemical modification. Conversely, these studies may prove that the overriding effect of PEGylation allows in itself maximal retention of AChE in the circulation, circumventing the need for optimized post-translation processing of the enzyme.

In the present study, we conducted a series of experiments designed to probe the interrelationship between either AChE lysine contents or AChE differential post-translational processing and PEG-conjugation with regard to the overall pharmacokinetic profile of the protein product. The data resulting from this study are provided herein, and the implications of our findings on the large-scale production of rHuAChE for therapeutic use are discussed.

## METHODS

### PEG-conjugation reaction and analysis of the products

Attachment of PEG chains to primary amines in the AChE enzymes was performed using succinimidyl propionate activated methoxy PEG (SPA-PEG; Shearwater polymers, Inc.). Purified recombinant enzymes (10  $\mu$ M) was incubated with PEG-20000 at a ratio of 100:1 [PEG]<sub>0</sub>/[AChE primary amines]<sub>0</sub> in 50mM borate buffer pH 8.5 for 2 hours at room temperature. The modified products were dialyzed extensively against phosphate buffer saline (PBS) using cellulose acetate membranes (cutoff 50KDa; Nest Group Inc.). Samples of the proteins were resolved on 5% SDS-polyacrylamide gels. To reduce the interaction of PEG with SDS (Odom *et al.*, 1997), Tris-HCl concentration in the stacking gel was decrease by 25%, to 93.8mM and concentration of SDS in the electrode buffer was lowered to 0.05%. PEGylated proteins were visualized by Coomassie staining or by BaI<sub>2</sub> staining which allows visualization of PEG. Preservation of AChE enzymatic activity was monitored by activity measurements as described below.

### Enzyme activity

AChE activity was measured according to Ellman *et al.* (1961). Assays were performed in the presence of 0.5 mM acetylthiocholine, 50 mM sodium phosphate buffer pH 8.0 0.1 mg/ml BSA and 0.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid). The assay was carried out at 27°C and monitored by a Thermomax microplate reader (Molecular Devices).

### Pharmacokinetics in mice

Clearance experiments in mice (3 to 6 ICR male mice per enzyme sample) and analysis of pharmacokinetic profiles were carried out as described previously (Kronman *et al.*, 1995). The study was approved by the local ethical committee on animal experiments. Residual AChE activity in blood samples was measured and all values were corrected for background activity determined in blood samples withdrawn 1 hour before performing the experiment. The clearance patterns of the various enzyme preparations were usually biphasic and fitted to a bi-exponential elimination pharmacokinetic model ( $C_t = Ae^{-k\alpha t} + Be^{-k\beta t}$ ) as described previously (Kronman *et al.*, 1995). This model enables determination of the parameters A and B which represent the fractions of the material removed from the circulation in the first-fast and second-slow elimination phases respectively, and  $T_{1/2\alpha}$  and  $T_{1/2\beta}$  which represent the circulatory half-life values of the enzyme in the fast and slow phases. The pharmacokinetic parameters MRT (mean residence time, which reflects the average length of time the administered molecules are retained in the organism) and CL (clearance, which represents the proportionality factor relating the rate of substance elimination to its plasma concentration ( $CL = \text{dose/area under the concentration-time curve}$ ), (Rowland and Tozer, 1989) were independently obtained by

analyzing the clearance data according to a noncompartmental pharmacokinetic model using the WinNonlin computer program (Laub and Gallo, 1996).

### Sucrose gradient

*Sucrose density gradient centrifugation* - Analytical sucrose density gradient centrifugation was performed on 5-25% sucrose gradients containing 0.1M NaCl/50 mM sodium phosphate buffer pH 8.0. Centrifugation was carried out in an SW41 Ti rotor (Beckman) for 26h at 160000. Fractions of 0.2 ml were collected and assayed for AChE activity. Alkaline phosphatase was used as a sedimentation marker.

### Non-denaturing deglycosylation of AChE

Purified recombinant AChE (500µg of either the wild-type or the C-terminal truncated enzyme) was subjected to two consecutive treatments with 250mU of N-glycanase plus (Glyko Inc.) at room temperature for 24 hours. AChE was cleared from the N-glycanase enzyme and the removed glycan chains by purification on procaine-amid column as described before (Kronman *et al.*, 1992, 1995). The complete removal of the glycans from AChE was monitored by SDS-PAGE analysis. Using these mild conditions for glycan removal results in the full preservation of the enzymatic activity of AChE.

### MALDI-TOF Analysis of Basic Glycan Structures

- Release, recovery, purification and labeling of N-glycans:

N-glycans of purified enzyme preparations (~100 µg protein) were released by N-glycosidase-F (Glyko, USA) treatment as described before (Kronman *et al.*, 1992). Deglycosylated protein was removed by ethanol-precipitation and glycans were recovered and purified from the supernatant as described by Kuster *et al.*, 1997. To increase sensitivity (Anumula and Dhume, 1998; Okafo *et al.*, 1996, 1997) purified glycans were fluorescently labeled. Fluorescent labeling of purified glycans with 2-aminobenzamide (2-AB) was performed according to Bigge *et al.*, 1995, using a commercial labeling kit (Glyko, USA).

- Sialic acid removal:

Agarose-bound sialidase (0.04U, Sigma) was prewashed 5 times with water and incubated at room temperature for 16h with 2AB labeled N-glycans released from 1.5 -2.0 nmol AChE. Sialidase was removed by Eppendorf centrifugation. Desialylated N-glycans were vacuum dried, resuspended in 30 µl of water and stored at -20°C until use.

- Mass spectrometry: Mass spectra were acquired on a Micromass ToFSpec 2E reflectron time-of-flight (TOF) mass spectrometer. 2AB-labeled desialylated or 2AB-labeled esterified glycan samples were mixed with an equal volume of freshly prepared DHB (10mg/ml in 70% acetonitrile) and loaded onto the mass spectrometer target. Routinely, 1 µl and 1 µl of glycan samples diluted 1:10 in water were subjected to analysis. Dried spots were recrystallized by

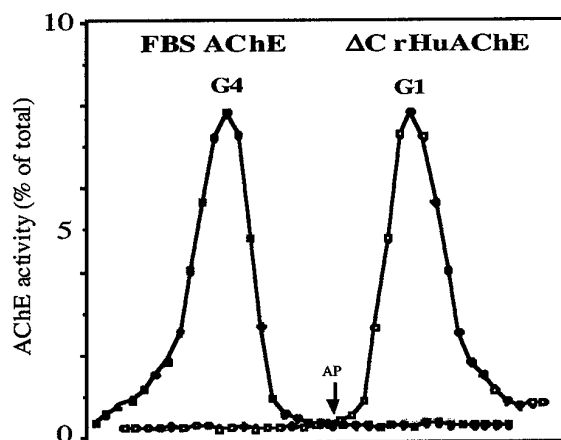
adding 0.5  $\mu$ l ethanol and allowed to redry. Neutral glycans were observed as  $[M+Na]^+$  ions. 1  $\mu$ l of peptide mixture (renin substrate, ACTH fragment 18-39, and angiotensin, 10pMole/ $\mu$ l, all from Sigma) which served as a three-point external calibrant for mass assignment of the ions was mixed with freshly prepared  $\alpha$ -cyano-4-hydroxycinnamic acid (10 mg/ml in 49.5% acetonitrile; 49.5% ethanol; 0.001% TFA), loaded on the mass spectrometer target and allowed to dry. All oligosaccharides were analyzed at 20 kV with a single-stage reflectron in the positive-ion mode. Between 100 and 200 scans were averaged for each of the spectra shown.



## RESULTS AND DISCUSSION

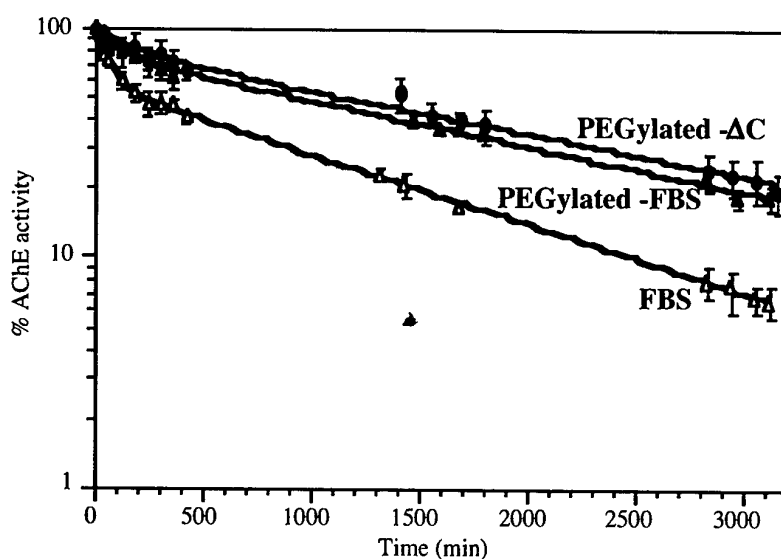
### Effect of enzyme subunit assembly on the circulatory retention of PEGylated rHuAChE

To examine whether the circulatory life-time of enzyme forms which are assembled into tetramers can be extended more efficiently by PEGylation than non-assembled enzyme forms, we compared the pharmacokinetic performance of PEGylated  $\Delta$ C-rHuAChE to that of native fetal bovine serum (FBS) AChE. The latter consists solely of tetramers, while the truncated rHuAChE enzyme is of monomeric nature in its entirety, as determined by sucrose gradient analysis (Fig. 1).



**Fig. 1: Sucrose-gradient sedimentation profiles of FBS-AChE and  $\Delta$ C-rHuAChE.** G4 indicates the sedimentation position of AChE tetramers and G1 indicates the sedimentation position of monomers. The arrow denotes the sedimentation position of the alkaline phosphatase marker (6.1 S) added to the samples.

The conjugation of PEG to FBS-AChE resulted in the generation of an enzyme form which is characterized by improved  $T_{1/2}$  and MRT values (Table 1) and resides in the circulation of mice for extended periods of time (Fig. 2), allowing us to conclude that PEGylation of serum-derived AChE can improve its circulatory residence. Yet these values were essentially the same as those determined for the PEGylated monomeric  $\Delta$ C-rHuAChE. Thus, although enzyme tetramerization has been shown in the past to contribute to the circulatory longevity of AChE (Kronman *et al.*, 2000; Chitlaru *et al.*, 2001), it does not provide an additional pharmacokinetic value to PEG-conjugation. We conclude therefore, that following PEG-conjugation, monomeric and tetrameric AChEs are retained in the circulation for extended periods of time in an equal manner.



**Fig. 2: Pharmacokinetic profiles of non-modified and PEG-modified FBS-AChE and PEG-modified  $\Delta$ C-rHuAChE.** The various exogenous rHuAChEs were introduced into mice at levels that were at least 30-fold higher than background levels. Residual AChE activity was determined in blood samples removed at various time periods.

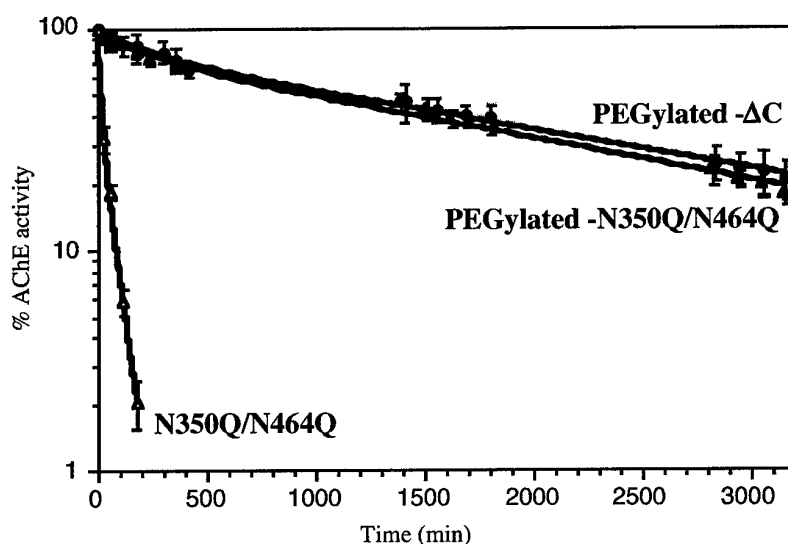
**Table 1: Pharmacokinetic parameters of non-modified and PEG-modified FBS-AChE and PEG-modified  $\Delta$ C-rHuAChE.**

AChE type	A (%)	B (%)	$T_{1/2\alpha}$ (min)	$T_{1/2\beta}$ (min)	MRT (min)
AChE-FBS	43 $\pm$ 2	57 $\pm$ 2	41 $\pm$ 5	990 $\pm$ 67	1390
PEGylated-FBS	21 $\pm$ 5	75 $\pm$ 6	65 $\pm$ 26	1535 $\pm$ 150	2065
PEGylated - $\Delta$ C	23 $\pm$ 4	76 $\pm$ 3	35 $\pm$ 15	1550 $\pm$ 120	2100

#### **Effect of glycan quantity and composition on the circulatory retention of PEGylated rHuAChE**

Unlike the human version of AChE, which contains three appended N-glycans (Velan *et al.*, 1993), the bovine enzyme carries a fourth oligosaccharide unit at amino acid 61 (Mendelson *et al.*, 1998). The finding that the circulatory lifetime of PEGylated FBS-AChE is similar to that of the C-terminal truncated version of the human AChE,  $\Delta$ C-rHuAChE, demonstrates that the

presence of an additional glycan unit in the bovine enzyme does not contribute to the ability of the PEG-conjugation process to extended circulatory residence. To further examine whether HuAChE forms carrying less than 3 N-glycans are negatively affected in their ability to reside in the circulation for extended periods of time following PEGylation, we monitored the pharmacokinetic behavior of the N350Q/N464Q rHuAChE mutant. The N-glycosylation consensus sequences at positions 350 and 464 of this enzyme form have been altered by Asn-to-Gln mutations, resulting in the generation of rHuAChE containing a single N-glycan unit at position 265 (Velan *et al.*, 1993). N350Q/N464Q rHuAChE is characterized by low circulatory retention ( $T_{1/2\beta}$  and MRT values = 37 and 50 mins, respectively, Table 2). PEG-conjugation of this hypoglycosylated AChE mutant resulted in a significant extension of its circulatory retention (Fig. 3 and Table 2). Most importantly, the pharmacokinetic values displayed by the hypoglycosylated enzyme did not differ substantially from those of the triglycosylated  $\Delta$ C-rHuAChE, ( $T_{1/2\beta}$  = 1540 and 1550 mins and MRT values = 2035 and 2100 mins, for the monoglycosylated and triglycosylated enzyme forms, respectively). Taken together, these results demonstrate that even low levels of enzyme glycosylation suffice for efficient circulatory lifetime extension of AChE, provided the enzyme is efficiently PEGylated.

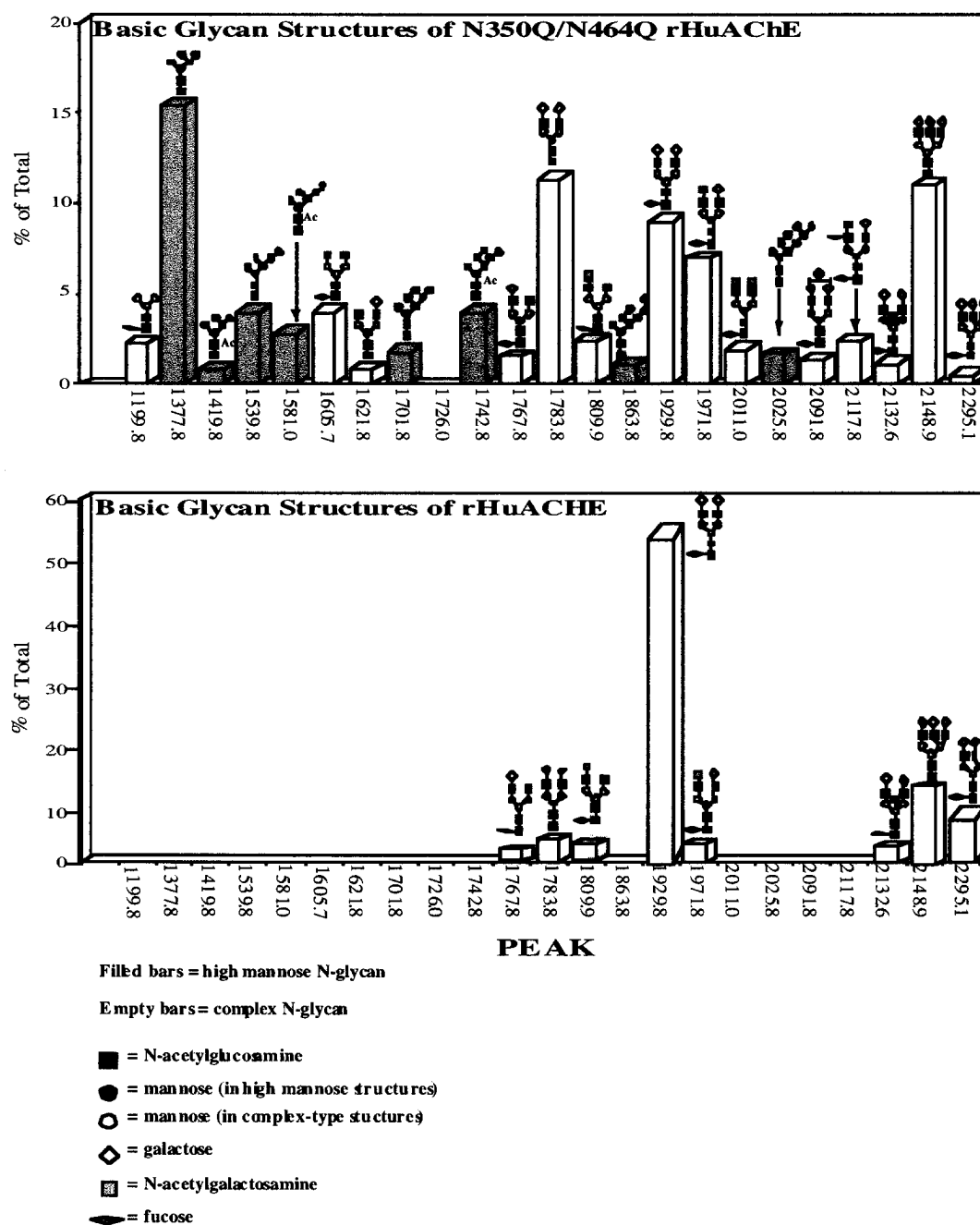


**Fig. 3: Pharmacokinetic profiles of non-modified and PEG-modified monoglycosylated N350Q/N464Q rHuAChE and PEG-modified  $\Delta$ C-rHuAChE.** The experiments were performed as detailed in the legend to Fig. 2.

**Table 2: Pharmacokinetic parameters of non-modified and PEG-modified monoglycosylated N350Q/N464Q rHuAChE and PEG-modified  $\Delta$ C-rHuAChE.**

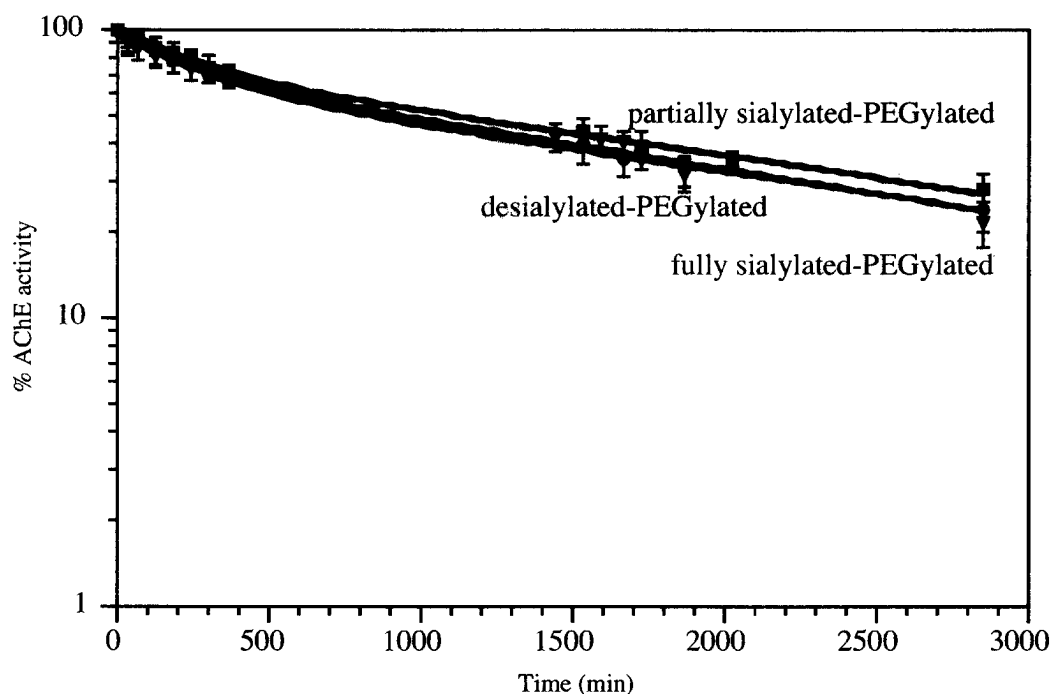
AChE type	A (%)	B (%)	$T_{1/2\alpha}$ (min)	$T_{1/2\beta}$ (min)	MRT (min)
AChE- N350Q/N464Q	43 $\pm$ 6	57 $\pm$ 5	3.5 $\pm$ 1	37 $\pm$ 3	50
PEGylated- N350Q/N464Q	21 $\pm$ 6	79 $\pm$ 5	25 $\pm$ 13	1540 $\pm$ 110	2035
PEGylated - $\Delta$ C	23 $\pm$ 4	76 $\pm$ 3	35 $\pm$ 15	1550 $\pm$ 120	2100

Examination of the basic glycan forms appended to the hypoglycosylated N350Q/N464Q rHuAChE by MALDI-TOF analysis, displayed that this enzyme form differs from wild-type HuAChE not only in its glycan quantity, but also in the actual glycan structures, which it contains (Fig. 4). While the wild-type enzyme carries an array of oligosaccharides consisting only of complex-type glycan forms, the monoglycosylated rHuAChE mutant contains glycans of both complex and high-mannose type. The high-mannose glycans form a series, which differ one from another in the number of mannose residues per glycan unit, ranging from 5 mannose/glycan (MW = 1377.8 Da) to 9 mannose/glycan (MW = 2025.8 Da), and those containing 5 to 7 mannose units appear in both non-acetylated and acetylated versions (Fig. 4, upper panel). In addition, the monoglycosylated rHuAChE displays (Fig. 4, upper panel) unique complex-type N-glycans, which were not detected in the wild-type enzyme or in other rHuAChE or rBoAChE forms examined previously (Kronman *et al.*, 2000; Chitlaru *et al.*, 2001,2002). These include glycan structures that carry 2 GalNac (MW = 2011.0 Da) or 2 fucose (MW = 2117.8 Da) moieties. It therefore appears that the severe reduction in the number of appended N-glycans causes the hypoglycosylated rHuAChE to undergo a different pattern of oligosaccharide processing within the cells. The finding that PEGylation of the hypoglycosylated rHuAChE leads to the generation of an enzyme form which is retained for periods of time in a manner similar to that of  $\Delta$ C-rHuAChE, confirms that PEG appendage can not only efficiently extend the circulatory residence of enzyme forms which carry significantly lower amounts of appended glycans, but that PEGylation can also extend the circulatory life-time of enzyme forms displaying altered glycan structures.



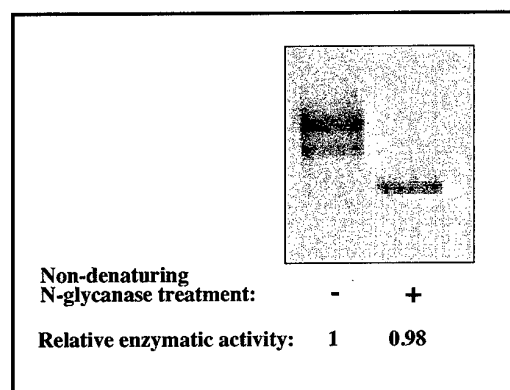
**Fig. 4: The basic structures and relative abundances of desialylated N-glycans released from monoglycosylated N350Q/N464Q rHuAChE and from wild-type rHuAChE.** Purified N-glycans released from the two enzyme forms were subjected to sialidase treatment and 2-AB labeling before MALDI-TOF analysis. Molecular masses and schematic structures are shown for the various glycan forms. Molecular weights represent monoisotopic masses of the respective  $[M+Na]^+$  ions of the glycan species.

Since our previous studies have shown that circulatory longevity of AChE is influenced mainly by the level of sialic acid capping of the glycans (Kronman *et al.*, 2000; Chitlaru *et al.*, 2001) rather than by their basic structures, we extended these studies to examine whether differences in sialic acid occupancy affect the ability to prolong circulatory retention by PEGylation of AChE. This was achieved by using three different preparations of the C-terminal truncated  $\Delta$ C-rHuAChE: (1) Partially sialylated enzyme produced in non-modified HEK-293 cells; (2) Fully sialylated  $\Delta$ C-rHuAChE produced in the genetically modified 2D6ST cell line, which coexpress high levels of  $\alpha$ -2,6 sialyltransferase (Chitlaru *et al.*, 1998, 2001), and (3) Desialylated  $\Delta$ C-rHuAChE, generated by subjecting the rHuAChE to sialidase treatment. All three enzyme forms were PEGylated and subjected to pharmacokinetic studies in mice. As shown in Fig. 5, desialylated, partially sialylated and fully sialylated  $\Delta$ C-rHuAChEs which exhibit mean residence time (MRT) values of 4, 42 and 163 minutes, respectively, are all characterized by MRTs of approximately 2000 minutes following PEGylation. Thus, the ability to confer extended circulatory longevity to  $\Delta$ C-rHuAChE by PEG conjugation is not affected by differences in levels of sialic acid capping.



**Fig. 5: Pharmacokinetic profiles of PEG-modified  $\Delta$ C- rHuAChE containing different levels of sialic acid.** The PEGylated rHuAChEs were introduced into mice at levels that were at least 30-fold higher than background. Residual AChE activity was determined in blood samples removed at various time periods.

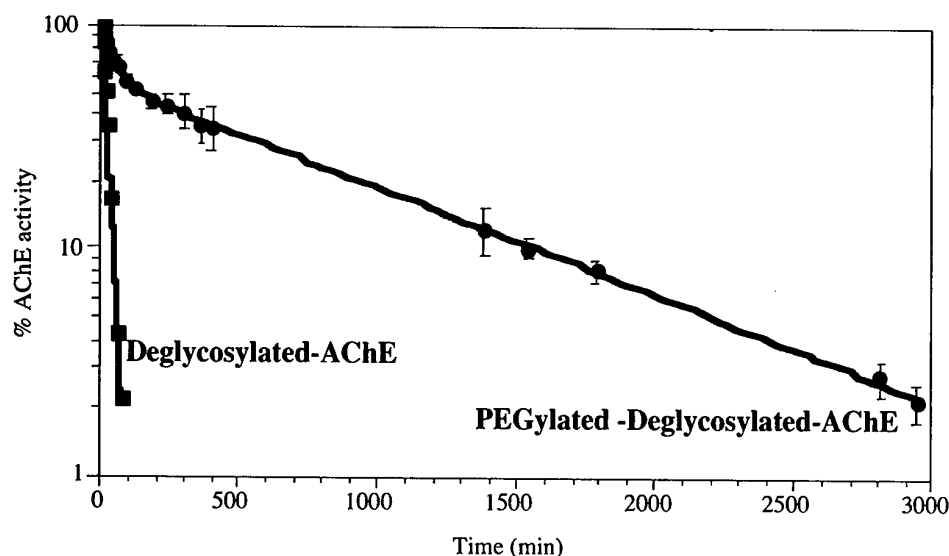
Since even low levels of enzyme glycosylation suffice for efficient circulatory lifetime extension of AChE by PEGylation, as manifested by the finding that PEG-conjugation of the AChE mutant carrying a single N-glycan unit at amino acid 265 resulted in a significant extension of its circulatory retention, we asked whether a minimal level of glycosylation is required at all for extension of circulatory residence by PEG conjugation. To answer this question we set out to monitor the pharmacokinetic performance of PEGylated deglycosylated-AChE. Since the efficient removal of N-glycans normally requires the full denaturation of the substrate protein prior to glycan removal, this method of generation of AChE totally devoid of glycans is not suitable for the monitoring of the residual enzymatic activity of the enzyme in the bloodstream of the administered mice. We therefore performed the enzymatic removal of AChE glycans under a different set of conditions, to allow glycan removal from enzyme preparations in their native conformation. This was achieved by incubating the hypoglycosylated N350Q/N464Q AChE in the presence of highly concentrated N-glycanase at room temperature for 48 hours, without prior heat denaturation.



**Fig. 6: SDS-PAGE analysis of  $\Delta$ C-HuAChE before and after non-denaturing N-glycan treatment.** N-glycans were enzymatically removed from  $\Delta$ C-rHuAChE as described in the text. Relative enzymatic activities before and after N-glycan removal are indicated.

This procedure resulted in the generation of AChE in which enzymatic activity is fully preserved, while SDS-PAGE analysis demonstrated that the N-glycanase-treated enzyme appears as a single faster-migrating band, attesting to the fact that glycans were indeed efficiently removed from the non-denatured enzyme (Fig. 6). This deglycosylated AChE was subjected to PEG-conjugation, and the chemically modified enzyme was administered to mice to evaluate its pharmacokinetic performance (Fig. 7). As in the case of glycan-bearing enzyme species, the circulatory retention of the deglycosylated AChE was significantly extended by PEG appendage, displaying a mean residence time (MRT) value of  $1910 \pm 75$  mins. This value, is very similar to that observed for the wild-type triglycosylated AChE (MRT = 2100 mins.)

Thus, the ability to extend the circulatory residence time of HuAChE by PEG, is not dependent on the presence of N-glycans, and can be effectively carried out using AChE, which is totally devoid of oligosaccharide appendages.



**Fig. 7: Pharmacokinetic profiles of non-modified and PEG-modified  $\Delta$ C-rHuAChE devoid of N-glycans.** N-glycans were enzymatically removed from  $\Delta$ C-rHuAChE as described in the text. The non-modified and PEGylated deglycosylated rHuAChEs were introduced into mice at levels that were at least 30-fold higher than background. Residual AChE activity was determined in blood samples removed at various time periods.

Taken together, this series of experiments leads to the conclusion that various post-translational modification-related characteristics of AChE such as its state of assembly, alterations in N-glycosylation levels or even their total absence, as well as variations in the basic structure and terminal capping of the appended glycans, do not affect the ability of the enzyme to undergo PEGylation in a manner which will allow it to reside in the circulation for extended periods of time. These findings are in line with the idea that protein PEGylation enhances circulatory longevity by forming a shielding cloud around the modified protein which thereby prevents access to receptors involved in protein clearance, and by increasing the overall molecular size of the modified protein and thus which preventing or decreasing its glomerular filtration (Monfardini and Veronese, 1998). These studies point towards the potential ability to utilize recombinant human AChE produced in bacterial production systems, and therefore lack all eukaryotic-related post translation modifications, as a cost-effective source for large-scale amounts of enzyme which can be thereby be efficiently PEGylated to generate circulatory long-lived enzyme species for therapeutic use.

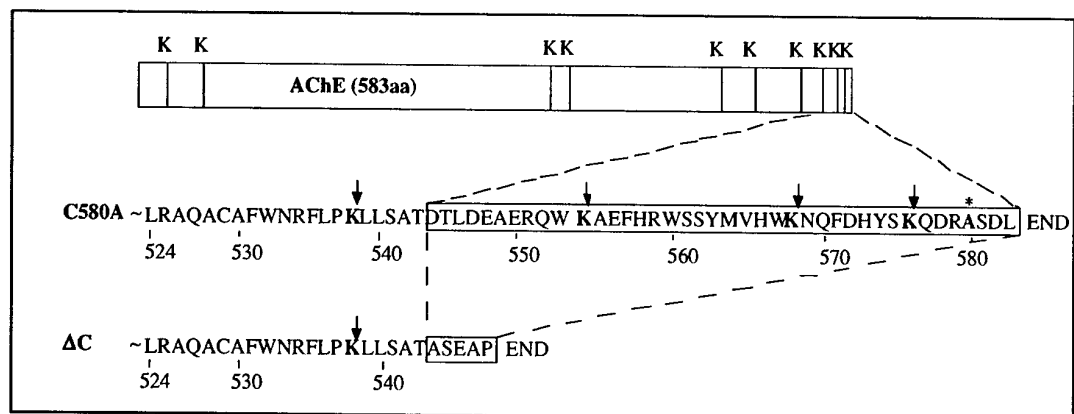


### Effect of lysine content on the circulatory retention of PEGylated rHuAChE

Three major points of consideration in the conversion of AChE into PEG-modified long-lived molecules which can serve as therapeutic bioscavengers are that the chemically modified product will: (i) reside in the circulation for sufficiently long-periods of time, (ii) retain its biological activity and, (iii) exhibit maximal homogeneity. To meet these requirements, the number of target sites available on AChE for PEG-conjugation may need to be modulated by either the reduction or addition of lysine residues. Human AChE contains ten lysine residues, three of which are located at the C-terminal end of the enzyme, while the other seven lysines are situated on the enzyme surface. The presence of ten lysines in human AChE which can potentially undergo PEGylation, may lead to the formation of mixed populations of enzyme forms comprised of a wide spectrum of differently modified forms. This can be alleviated in part by the utilization of the C-terminal truncated version of the enzyme (Kryger *et al.*, 2000) which is devoid of three lysine residues situated at the C-terminal portion of the enzyme. Increased homogeneity may be further achieved by the stepwise mutation of some of the lysine residues. This mode of operation should take into account the gain and loss in terms of the pharmacokinetic performance of enzyme which is PEGylated at a lower extent. In any case, removal of selected lysine residues should be restricted to those which are not required for the catalytic activity of the enzyme, and which do not play a role in the reactivity of the enzyme towards organophosphates. This can be readily evaluated by the generation of an assortment of AChE molecules, each of which is devoid of a particular lysine moiety. This array of lysine-deficient mutants can then be evaluated for their catalytic properties, to determine the set of critical lysines which are not to be eliminated from the molecule. Conversely, under optimized PEGylation conditions, the generation of fully PEGylated bioactive AChE may yet be restrained by the deleterious effect of PEG-appendage at specific lysine residues. For example, PEGylation of the lysine at position 348 of human AChE, which is located at the entrance to the gorge leading to the catalytic site of the enzyme, may prove to sterically hinder the diffusion of substrate to the active center. If this is indeed the case, such a lysine residue should be substituted by a different amino acid to prevent PEG appendage. Determination of crucial lysine residues which cannot be modified without compromising enzyme activity or reactivity to organophosphates, could be determined by the generation and catalytic study of an array of PEG-AChE molecules which carry different combinations of lysine residues. This line of study will allow the designation of specific lysine moieties whose replacement is required for the generation of circulatory long-lived PEG-AChE which retains its biological activity.

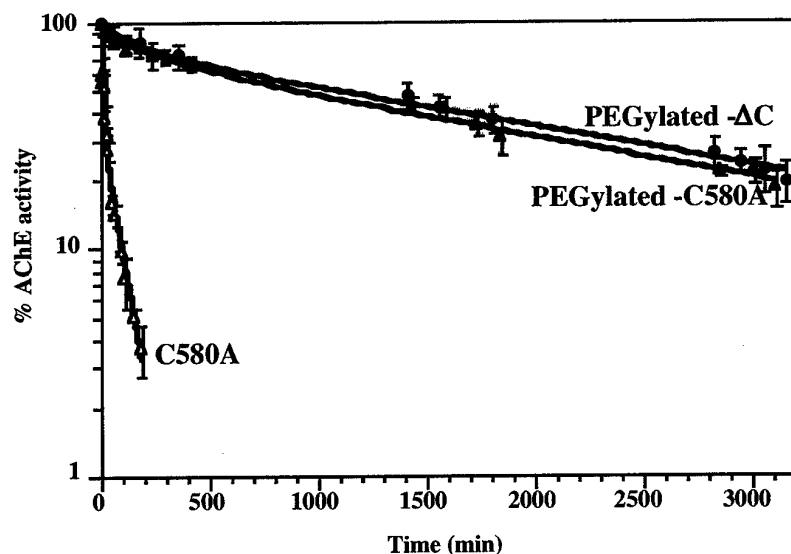
To determine whether the pharmacokinetic performance of PEGylated rHuAChE is affected by the number of sites available for PEG-appendage, we compared as a first step, the pharmacokinetic profiles of PEGylated mutated rHuAChE species in which either cysteine 580 was substituted by alanine (C580A, Velan *et al.*, 1991), or the C-terminal portion of the enzyme corresponding to amino-acids 544 to 583 was deleted ( $\Delta$ C-rHuAChE, Kryger *et al.*, 2000).

Both of these enzyme forms are impaired in their ability to form dimers due to the absence of cysteine 580, involved in interchain disulphide bonding. The two monomeric AChE forms differ however one from another in the number of lysine residues available for PEG conjugation. While the C580A AChE carries 10 lysines, the  $\Delta$ C-rHuAChE C-terminus-truncated enzyme contains only 7 (Fig. 8).



**Fig. 8: Schematic representation of the distribution of lysine residues along the HuAChE sequence and the C-terminal sequence of the C580A and  $\Delta$ C-rHuAChE forms. Lysine residues are marked by arrows. The C580A mutation is marked by asterisk.**

The two enzyme forms were conjugated to PEG (approx. MW =20,000) using succinimidyl-propionate-activated PEG, which specifically targets the PEG moieties to lysine residues. The chemically modified preparations were administered to mice, residual AChE activity was monitored in blood samples removed at various periods of time, and the elimination curves of the two enzyme forms were plotted (Fig. 9).



**Fig. 9: Pharmacokinetic profiles of non-modified and PEG-modified C580A rHuAChE and PEG-modified  $\Delta$ C-rHuAChE.** The experiments were performed as detailed in the legend to Fig. 2.

**Table 3: Pharmacokinetic parameters of non-modified and PEG-modified C580A rHuAChE and PEG-modified  $\Delta$ C-rHuAChE.**

AChE type	A (%)	B (%)	$T_{1/2\alpha}$ (min)	$T_{1/2\beta}$ (min)	MRT (min)
AChE-C580A	65 $\pm$ 3	35 $\pm$ 2	4 $\pm$ 1	50 $\pm$ 8	73
PEGylated-C580A	19 $\pm$ 5	75 $\pm$ 7	55 $\pm$ 16	1590 $\pm$ 150	2085
PEGylated - $\Delta$ C	23 $\pm$ 4	76 $\pm$ 3	35 $\pm$ 15	1550 $\pm$ 120	2100

The two PEGylated rHuAChEs, C580A and  $\Delta$ C- rHuAChE, displayed nearly identical pharmacokinetic profiles, characterized by very similar half-life and mean residence time values (Table 3). These findings demonstrate that the variance in number of accessible lysines in the two enzyme preparations (10 versus 7 for the C580A and  $\Delta$ C- rHuAChEs, respectively) does not affect the ability of the enzyme to undergo efficient PEGylation. We note however that this apparent insensitivity to lysine number may simply reflect the fact that in both of the enzyme species under examination, the lysine contents exceeds the maximal amount required for optimal

circulatory retention, and that enzyme species characterized by lower lysine contents may display differential circulatory longevity following PEGylation. This issue, which may have important implications on the generation of homogenously PEGylated enzyme forms of lower lysine content, will be further addressed by monitoring the pharmacokinetic performance of PEG-conjugated hypolysine AChE species. To this end, we generated to date, a series of hypolysine AChEs lacking one or two lysine residues. These hypolysine mutants are now being assessed for their catalytic activity and thermostability. Based on these studies, multilysine mutants which display normal catalytic activity and thermostability will be generated, subjected to PEGylation and evaluated for their pharmacokinetic performance.

### **III. Generation of PEG-Conjugated rHuAChE Devoid of Mammalian Post-Translation Modifications - Bacterially Expressed Synthetic rHuAChE Gene**

#### **INTRODUCTION**

As documented at length in the previous section, optimized PEG-conjugation results in the long-term retention of AChEs in the circulation even when these are not optimally processed. Thus, proteins lacking glycan moieties or containing glycans which are not capped by sialic acid, exhibit extended circulatory retention following PEG-conjugation, probably due to the masking effect of the appended PEG which obstructs removal via receptor-dependent elimination systems. Likewise, assembly of AChE into tetramers is not a prerequisite for circulatory longevity, most likely since the molecular size of the PEGylated enzyme decreases or prevents clearance through glomerular filtration. Although AChE forms differing in their number of glycans, level of glycan sialylation and state of assembly can serve for the investigation of the effect of different levels of post-translation processing on the circulatory longevity of PEGylated AChE molecules, extension of these studies for the determination of the effect of different levels and extent of PEG-conjugation on the circulatory behavior of AChE molecules devoid of any animal cell-related post-translation modifications, will require the study of AChE produced in microorganism-based expression systems.

Generation of human acetylcholinesterase in an *E. coli*-based production system was documented in the past (Fischer *et al.*, 1993). However, the recombinant enzyme produced in this system segregated as misfolded protein forms in insoluble inclusion bodies, and only 3% of the recombinant AChE could be successfully refolded into enzymatically active forms. Different approaches were explored in the past for the prevention of inclusion body segregation of recombinant proteins in *E. coli*. These included expression of the protein of interest fused to a solubilizing protein, targeting of the recombinant protein to the periplasm, coexpression of chaperones together with the protein of interest, and facilitation of disulfide bond formation in the cytoplasm. However, each of these strategies had serious drawbacks. The use of fused proteins for the generation of soluble recombinant bioactive products was complicated by the need for enzymatic cleavage which resulted in non-specific cleavage within the product protein, while the presence of highly bioactive proteases requires additional purification steps, increase production costs and can prohibit drug approval. Targeting of recombinant proteins to the oxidizing environment of the periplasm to allow their correct folding, proved to be a particularly complex and incompletely understood process (Pugsley, 1993), while the presence of a targeting signal peptide did not always ensure efficient protein translocation through the inner membrane. Coexpression of chaperones for the solubilization of recombinant proteins was complicated by the fact that (i) the correct substrate-chaperone combination has to be found by trial and error, (ii) overexpression of chaperones led in some cases to undesirable phenotypes

that were detrimental to viability and protein expression (Blum *et al.*, 1992) and (iii) the increase in yield of properly folded proteins as a result of chaperone coexpression proved to be inconsistent (Wall and Pluckthun, 1995; Yasukawa *et al.*, 1995; Makrides, 1996).

*Bacillus* species synthesize and secrete many extracellular enzymes directly into the medium in high yields and therefore serve as an attractive alternative to *E. coli*, for expression and secretion of biotechnologically important proteins (Simonen and Palva, 1993). The successful production/secretion of high levels of uniformly processed recombinant product in *Bacillus* requires the introduction of specific modifications both in the expression vector and in the host strain which will result in a favorable combination of biochemical and genetic traits. For instance, proteases of various species of *Bacillus* may severely affect production and secretion of foreign proteins by these bacteria. To remedy this situation, mutant strains that produce less proteases were generated (Sloma *et al.*, 1990; Wu *et al.*, 1991; Ye *et al.*, 1999; Lee *et al.*, 2000). Progressive mutagenesis of *Bacillus subtilis* resulted in the generation of strains which are deficient in two (subtilisin and neutral protease, the major *B. subtilis* proteases), five, six and seven proteases. The latter strain, WB700, allowed 8-fold higher production/secretion of staphylokinase, than wild type *B. subtilis* (~340mg/ml under optimized conditions, Ye *et al.*, 1999). An alternative approach to overcome the problem of extensive proteolytic degradation, is to use *B. brevis* rather than *B. subtilis*. This bacillus strain naturally secretes very low amounts of proteases, or none at all. For example, extracellular protease activity of *B. brevis* 47 is 1.6% of that of *B. subtilis*, and that of *B. brevis* HPD31 is below detection level (Tagaki *et al.*, 1989). Several foreign proteins such as *B. stearothermophilus*  $\alpha$ -amylase, swine pepsinogen, human epidermal growth factor and human interleukin-6, have been successfully produced by using these two *B. brevis* strains as hosts (Tsukagoshi *et al.*, 1985; Takao *et al.*, 1989; Udaka *et al.*, 1989; Yamagata *et al.*, 1989; Sagiya *et al.*, 1994; Ebisu *et al.*, 1996; Nagao *et al.*, 1997; Shiga *et al.*, 2000).

In recent years high level production and extracellular secretion of recombinant enzymes were achieved in *Bacillus*-based systems using different combinations of parameters which affect production. In most cases the host strain were either the six or seven protease-deficient strains of *B. subtilis* (WB600, WB700, W751) or low protease strains of *B. brevis* (HPD31, *B. brevis* 47). In some cases, the signal peptide of the protein of interest was modified to comply with the general structure of *Bacillus* signal peptides, but in most cases the original signal peptide was replaced with a *B. subtilis* or *B. brevis* signal. Likewise, the promoter driving transcription was usually of *Bacillus* origin (e.g. Pamy of *B. amyloliquefaciens*, P43 of *B. subtilis*, cell wall protein promoter of *B. brevis*). Extensive studies designed to find an optimal combination of host cells (*B. subtilis* vs. *B. brevis*, examination of specific strains), signal-peptide, signal-protein joining procedure, and transcription promoter, led to high-level production and secretion of recombinant proteins within the 200-1000 mg/liter range. In the case of recombinant human epidermal growth factor produced in a *B. brevis*-based system, large-scale production levels reached up to 1.5 gram per liter (Ebisu *et al.*, 1996).

The successful production of rAChE in a microorganism-based expression system will allow precise evaluation of the contribution of PEGylation to the circulatory longevity of AChE completely devoid of animal cell-dependent post-translation modifications. Furthermore, the development of microorganism-based expression systems may become useful also for cost-effective production of rAChE for the generation of circulatory long-lived PEGylated enzyme. In this section, we summarize the work carried out in our laboratory during the past year, aimed at the production of recombinant human AChE in microorganism-based systems.

## METHODS

### Planning and construction of a synthetic human AChE gene

Analyses of nucleotide frequencies, codon usage, restriction site contents and potential RNA secondary structures, were carried out using several programs in the GCG Wisconsin software package (Accelrys Inc.) including StemLoop and mFold softwares.

For the assembly of the synthetic gene, 46 oligonucleotides (65-83 bases) were synthesized by Sigma Inc., Israel. Oligonucleotides were resolved on urea-PAGE gels, extracted with butanol and purified on G-50 columns. Phosphorylation of the 5' ends of inner oligonucleotides was carried out using T4 kinase (BioLab, Inc.). Pairs of oligonucleotides were denatured at 95°C, annealed at 42°C and ligated overnight at 4°C with T4 ligase. Ligated oligonucleotides were mixed, denatured and allowed to re-ligate, to form larger segments corresponding to the N-terminal, mid-gene, and C-terminal regions of the synthetic gene, which were then amplified by PCR. The PCR products were resolved and excised from a 1% agarose gel, cut with restriction enzymes (SacI and BstEII, BstEII and EcoRV, EcoRV and BamHI for the first, second and third segments respectively) and ligated to pGEM3Z vector. The segments were assembled in tandem to generate the full-length coding region of the synthetic human AChE gene in the pGEM vector.

The synthetic AChE coding region and the upstream *B. brevis* ribosome binding site and signal-peptide were completely sequenced by ABI310 genetic analyzer using T7, SP6 and inner primers. Erroneous codons were replaced by proper codons by site-directed mutagenesis using the Quickchange system (Stratagene, Inc.)

### *In-vitro* expression system

The pGEM-sAChE plasmid (1µg) was subjected to <sup>35</sup>S-methionine labeled *in-vitro* transcription-translation (TNT Quick Coupled Transcription/Translation Systems, Promega Inc.). The reaction products were immunoprecipitated with polyclonal mouse anti-HuAChE (Shafferman *et al.*, 1992) antibodies, or with non-related control anti-GFP antibodies and protein G agarose beads (Sigma Inc.). Immunoprecipitated proteins were resolved by SDS-PAGE and visualized by fluorography (Amplify, Amersham Biosciences).



## RESULTS

### Construction of a Human AChE Synthetic Gene

Comparison between the human AChE gene and the genomes of *E. coli*, *B. subtilis*, *P. pastoris* and *S. cerevisiae* (Nakamura *et al.*, 2000) demonstrates that while the genomes of these microorganisms display an average GC content of 45%, the coding sequence for  $\Delta$ C-HuAChE gene is characterized by a significantly higher GC content of 65.2%. In fact, the GC content of the first 100 base pairs of the coding sequence for  $\Delta$ C-HuAChE gene reaches even higher values of 72%. Codon usage in  $\Delta$ C-HuAChE also differed significantly from that of microorganisms such as *Bacillus* or *Pichia* (see Table 4 A,B; Fig. 10A).

To allow optimal expression of human AChE in microorganisms, we set out to design a synthetic HuAChE gene (sAChE) of lower GC content, which codes for authentic human AChE, utilizing nucleotide codons which will be compatible with efficient expression in microorganisms-based systems.

Meticulous planning led to the proposal of a candidate nucleotide sequence coding for human AChE in which: (1) The overall GC content is 51.7%, as opposed to 65.2% in the original  $\Delta$ C-HuAChE gene (Fig. 10B); (2) The probability of generation of RNAs with stable secondary structures, which might have an unfavorable effect on the translation process is considerably lower; (3) Additional unique restriction sites for future manipulation of individual lysine residues have been introduced, and (4) Codon usage conforms to that of microorganisms such as *Bacillus* or *P. pastoris* to a much greater extent (Table 4 A, B, C, Fig. 10A). For example, the usage frequency values of codons CCC(Pro), GTG(Val), GCC(Ala), CTG(Leu), which in the native HuAChE sequence are 44-79 per 1000 nucleotides, are now reduced to 5.5-22 per 1000 nucleotides, in a manner similar to that displayed by various microorganisms. Similarly, codons displaying in the native HuAChE coding sequence very low usage frequency values of 0-5 per 1000 nucleotides, such as AGA(Arg) and TTA(Leu), and GTT(Val), were elevated in the synthetic coding region to 18-31 per 1000 nucleotides, conforming with their usage in microorganisms (Fig. 10A).

To construct the modified human AChE gene (synthetic rHuAChE), we synthesized 46 DNA oligonucleotide fragments (65 to 83 nucleotides each), spanning the full length of both strands of the synthetic rHuAChE. Oligonucleotide fragments corresponding to the ribosome binding site and signal peptide of the *B. brevis* cell wall protein (CWP, Tsuboi, *et al.*, 1986; Yamagata *et al.*, 1987) were synthesized as well. The oligonucleotides were grouped according to their location within the gene (N-terminal and *B. brevis* sequences, mid-gene, C-terminal), and oligonucleotide members of each group were joined one to another in a stepwise manner, as depicted in Fig. 11. Briefly, phosphorylated oligonucleotides corresponding to contiguous sequences were allowed to anneal and were then ligated. Nearest neighbor ligation products (comprised of 4 oligonucleotides) were then paired and subjected to a second round of annealing/ligation. These were then joined in a similar manner to form a segment corresponding to a one third length of the synthetic AChE gene. The three double stranded products

corresponding to the one-third *ache* gene segments, were then amplified by PCR, and the PCR products were cloned into the pGEM3 vector. These segments, which also include a *B. brevis* ribosome-binding site and signal peptide, were assembled in tandem to generate the full-length coding region of the synthetic human AChE gene (Fig.12A). Sequence verification of the synthetic AChE coding region allowed us to determine that three of the codons did not code for correct human AChE amino acids. These erroneous codons were exchanged with the proper codons by site-directed mutagenesis using the Quickchange system (Stratagene, Inc.).

**Table 4: Comparison of the codon usage of native rHuAChE to that of several microorganisms and the synthetic HuAChE**

A.

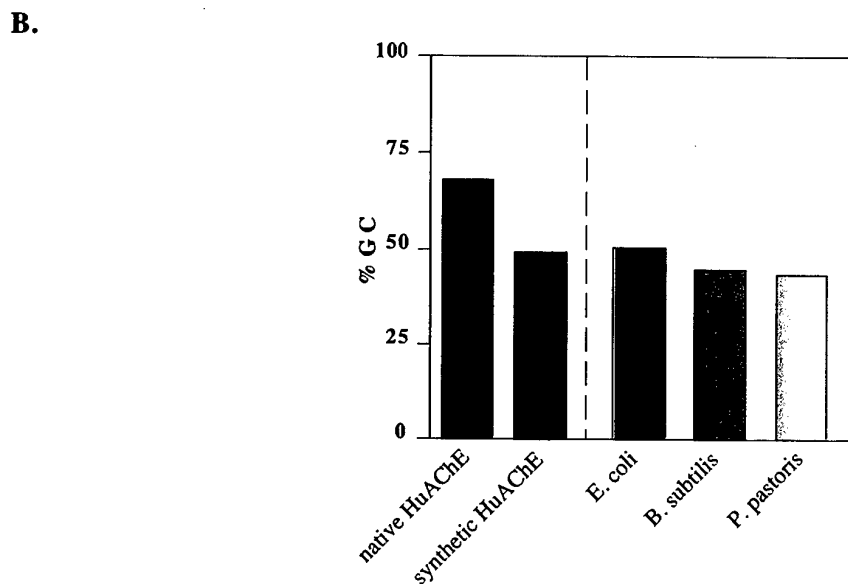
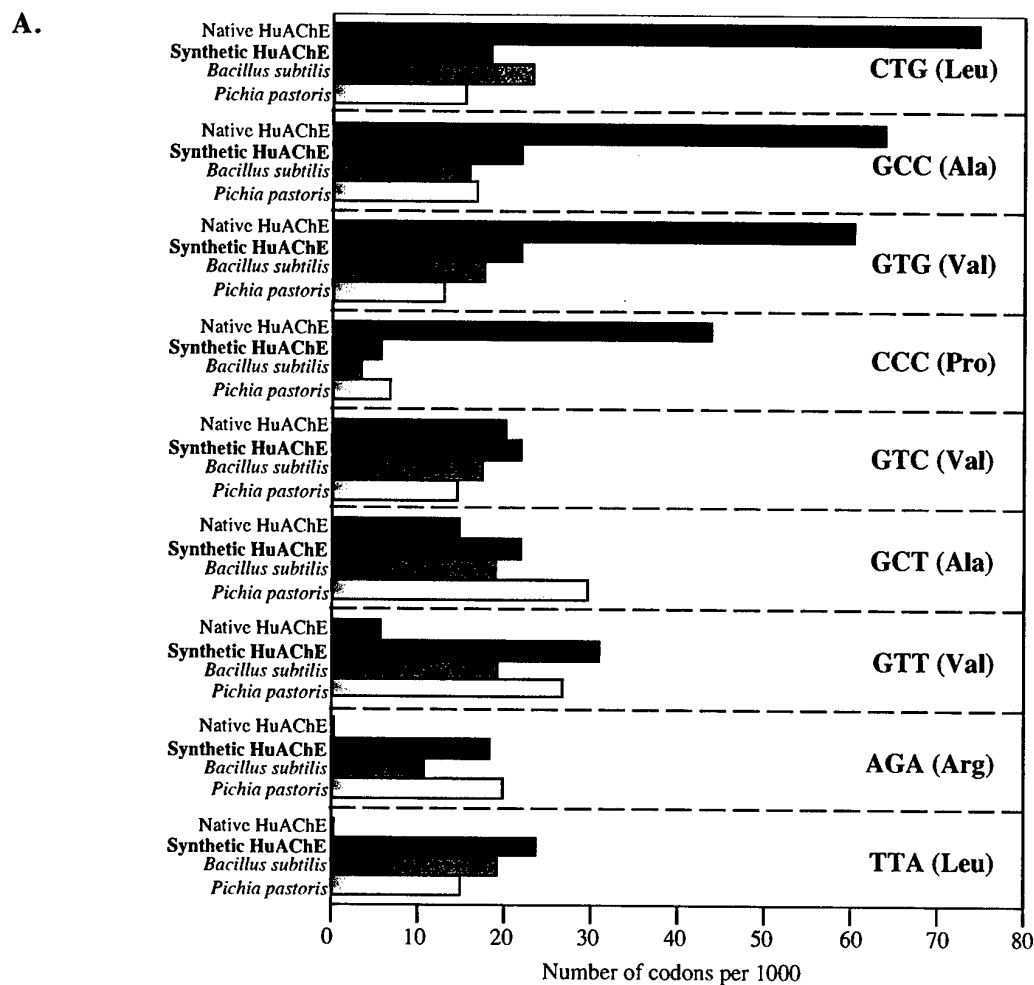
Nucleotide triplet		Codons in native HuAChE		Codons in <i>B. subtilis</i>											
		Amino-acid													
TTT	Phe	16.4	30.2	TCT	Ser	5.5	12.9	TAT	Tyr	5.5	22.6	TGT	Cys	1.8	3.6
TTC		32.9	14.2	TCC		14.6	8.1	TAC		29.2	12.0	TGC		9.1	4.3
TTA	Leu	0	19.1	TCA		3.7	14.8	TAA	Stop			TGA	Stop		
TTG		5.5	15.3	TCG		5.5	6.4	TAG				TGG	Trp	23.7	10.3
CTT		3.7	23.0	CCT	Pro	12.8	10.6	CAT	His	3.7	15.2	CGT	Arg	9.1	7.6
CTC		16.4	10.8	CCC		43.8	3.3	CAC		16.4	7.5	CGC		14.6	8.6
CTA	Ile	1.8	4.9	CCA		16.4	7.1	CAA	Gln	5.5	19.7	CGA		12.8	4.1
CTG		74.8	23.1	CCG		14.6	16.1	CAG		31.0	18.7	CGG		25.6	6.5
ATT	Ile	1.8	36.8	ACT	Thr	49.3	8.7	AAT	Asn	9.1	22.1	AGA	Ser	9.1	6.6
ATC		14.6	27.0	ACC		14.6	8.6	AAC		20.1	17.3	AGG		18.3	14.2
ATA		0	9.3	ACA		12.8	22.3	AAA	Lys	5.5	49.1	AGT	Ser	0	10.6
ATG	Met	12.8	26.9	ACG		14.6	14.6	AAG		7.3	20.8	AGC		9.1	3.9
GTT	Val	5.5	19.2	GCT	Ala	14.6	19.0	GAT	Asp	12.8	33.0	GGT	Gly	14.6	12.8
GTC		20.1	17.3	GCC		63.9	15.9	GAC		31.0	18.8	GGC		38.3	23.4
GTA		9.1	13.4	GCA		9.1	21.7	GAA	Glu	5.5	48.9	GGA		12.8	21.7
GTG		60.2	17.7	GCG		9.1	20.2	GAG		52.9	23.1	GGG		32.9	11.1

## B. Codons in native HuAChE

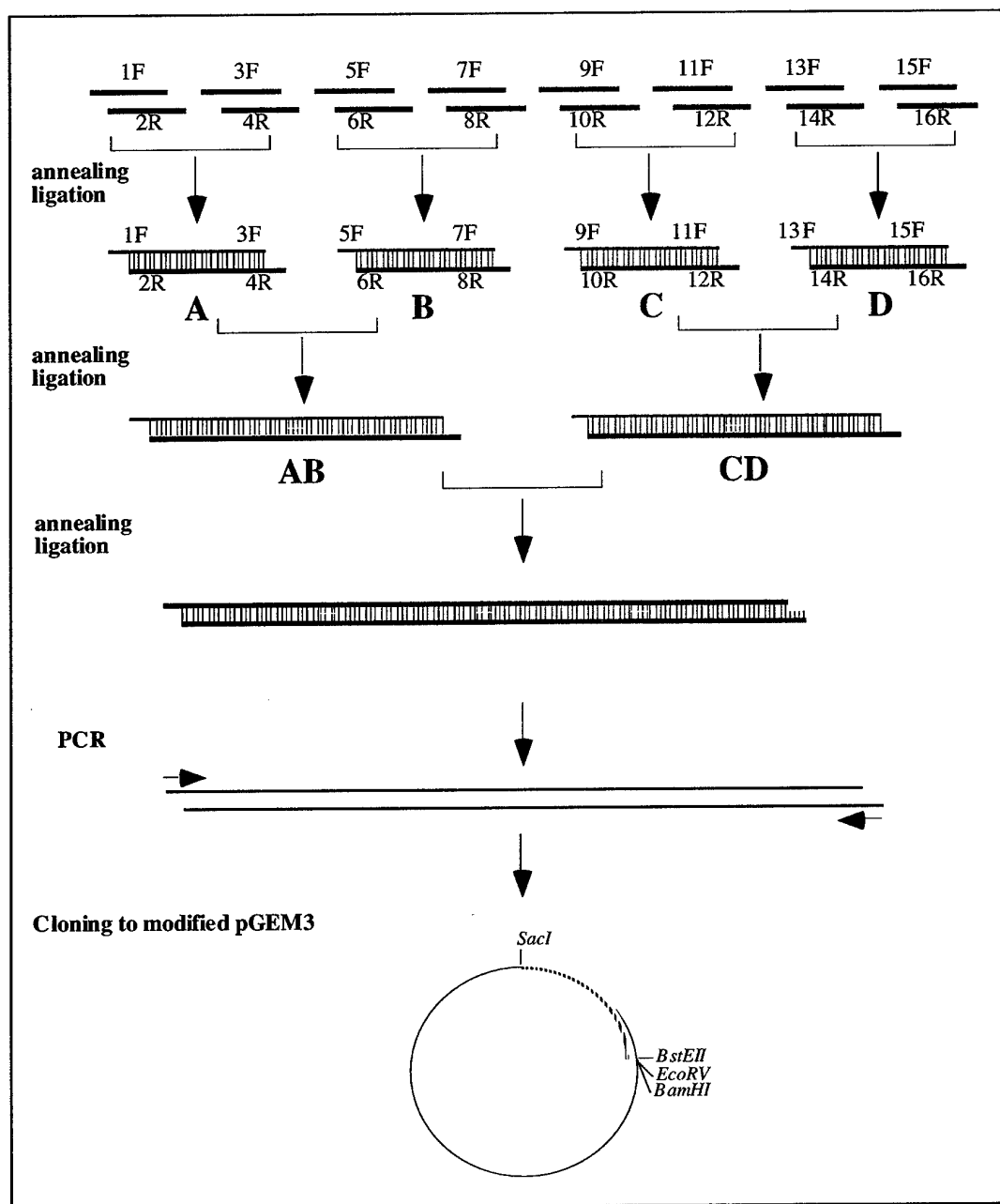
Nucleotide triplet	Amino-acid	Codons in native HuAChE	Codons in <i>P. pastoris</i>
TTT	Phe	16.4	38.3
TTC		32.9	11.0
TTA		0	23.7
TTG		5.5	16.4
CTT	Leu	3.7	29.2
CTC		16.4	7.3
CTA		1.8	7.3
CTG		74.8	18.3
ATT	Ile	1.8	7.3
ATC		14.6	5.5
ATA		0	3.7
ATG	Met	12.8	12.8
GTT		5.5	31.0
GTC	Val	20.1	21.9
GTA		9.1	20.1
GTG		60.2	21.9
TCT		5.5	9.1
TCC	Ser	14.6	5.5
TCA		3.7	12.8
TCG		5.5	7.3
CCT		12.8	27.4
CCC	Pro	43.8	5.5
CCA		16.4	27.4
CCG		14.6	27.4
TAT	Tyr	5.5	23.7
TAC		29.2	11.0
TAA	Stop		
TAG			
TGT			
TGC	Cys		
TGA	Stop		
TGG	Trp		
CGT			
CGC	Arg		
CGA			
CGG			
AGA			
AGG			
AGT	Ser		
AGC			
GGT			
GGC	Gly		
GGA			
GGG			

## C.

Nucleotide triplet	Amino-acid	Codons in native HuAChE	Codons in synthetic HuAChE
TTT	Phe	16.4	23.9
TTC		32.9	19.1
TTA		0	14.9
TTG		5.5	31.4
CTT	Leu	3.7	16.0
CTC		16.4	7.6
CTA		1.8	11.2
CTG		74.8	15.3
ATT	Ile	1.8	31.7
ATC		14.6	19.3
ATA		0	11.5
ATG	Met	12.8	19.2
GTT		5.5	26.7
GTC	Val	20.1	14.5
GTA		9.1	10.1
GTG		60.2	12.8
TCT		5.5	23.5
TCC	Ser	14.6	16.3
TCA		3.7	15.6
TCG		5.5	7.2
CCT		12.8	15.3
CCC	Pro	43.8	6.7
CCA		16.4	17.1
CCG		14.6	4.1
TAT	Tyr	5.5	14.7
TAC		29.2	18.3
TAA	Stop		
TAG			
TGT			
TGC	Cys		
TGA	Stop		
TGG	Trp		
CGT			
CGC	Arg		
CGA			
CGG			
AGA			
AGG			
AGT	Ser		
AGC			
GGT			
GGC	Gly		
GGA			
GGG			



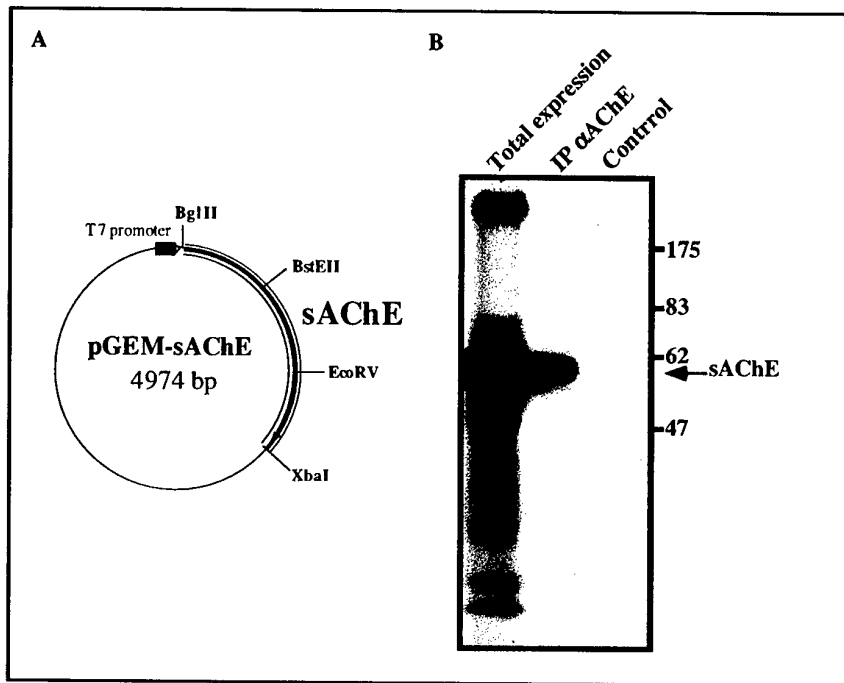
**Fig. 10: Representative codon usage (A) and GC content (B) of native rHuAChE, synthetic HuAChE and several microorganisms**



**Fig. 11: Schematic presentation of the assembly of the synthetic HuAChE gene.** Synthetic oligonucleotides of the N-terminal, mid-gene, or C-terminal segments of the HuAChE coding regions were assembled in a stepwise manner which included rounds of annealing and ligation, PCR amplification of the combined gene segment, and cloning of the PCR product into the pGEM3 vector. In the case of the N-terminal portion of the gene, oligonucleotides corresponding to the ribosome binding site and signal peptide of the *B. brevis* cell wall protein (CWP) were included for assembly upstream to the sAChE gene.

### Characterization of the Synthetic Human AChE Gene Product in an *in-vitro* Transcription-Translation System

To determine that the synthetic human AChE gene can indeed be translated into authentic AChE, the sAChE-containing pGEM3-based vector, which contains the T7 promoter upstream to the sAChE sequence, was subjected to radioactively labeled *in-vitro* transcription-translation (TnT system, Promega Inc.), followed by immunoprecipitation with polyclonal mouse anti-HuAChE antibodies. Immunoprecipitated protein was resolved by SDS-PAGE and visualized by fluorography. A single major protein product corresponding to full-length AChE was specifically precipitated by the anti-AChE antibodies (Fig. 12B), verifying that genuine human AChE enzyme was indeed generated.



**Fig. 12: Description of the assembled synthetic AChE gene and characterization of its protein product in an *in-vitro* transcription/translation system.** A. The three synthetic gene segments coding for the N-terminal, middle, and C-terminal regions of the human *ache* gene were assembled in tandem within the pGEM3 cloning vector, to generate the pGEM-sAChE plasmid, containing the full length synthetic *ache* gene, under control of the T7 promoter. B. The pGEM-sAChE plasmid was subjected to radioactively labeled *in-vitro* transcription-translation, followed by immunoprecipitation with polyclonal mouse anti-HuAChE antibodies, or with non-related control anti-GFP antibodies. Immunoprecipitated protein was resolved by SDS-PAGE and visualized by fluorography.

The synthetic human AChE gene will now be introduced into appropriate expression vectors, downstream to various *Bacillus*-oriented promoters, to allow human AChE production in *B. brevis*.

#### IV. REGULATION OF ACETYLCHOLINESTERASE CIRCULATORY RESIDENCE BY POST-TRANSLATIONAL PROCESSING AND SPECIES-SPECIFIC AMINO ACID EPITOPES

##### INTRODUCTION

Acetylcholinesterase plays a pivotal role in the cholinergic system where it functions in the rapid termination of nerve impulse transmission. AChE, unlike the related butyrylcholinesterase which is abundant in the serum of adult primates, is preferentially found in insoluble, cell-associated forms (Massoulie *et al.*, 1999). Previous studies suggest that exogenous cholinesterase can serve as an effective therapeutic agent in the prophylaxis and treatment of organophosphate (OP) poisoning. Indeed the successful exploitation of the scavenging potential of administered cholinesterase has been demonstrated in rodents and in non-human primates (Wolfe *et al.*, 1987; Broomfield *et al.*, 1991; Maxwell *et al.*, 1992; Raveh *et al.*, 1993). The use of human AChE as an efficient therapeutic bioscavenger has been advanced by the development of recombinant production systems (Kronman *et al.*, 1992; Lazar *et al.*, 1993) and the introduction of catalytically favorable mutations (Millard *et al.*, 1995; Ordentlich *et al.*, 1996; Shafferman *et al.*, 1996; Hosea *et al.*, 1996).

In addition to their high reactivity towards OP toxic agents, native serum-derived cholinesterases (serum butyrylcholinesterase or AChE from fetal bovine serum) were found to be retained in the circulation of experimental animals for extended periods of time, exhibiting mean residence times (MRTs) of more than 1000 minutes (Kronman *et al.*, 1995; Saxena *et al.*, 1997; Mendelson *et al.*, 1998; Saxena *et al.*, 1998). In contrast, recombinant human or bovine AChEs produced in stably transfected cell lines of the human embryonic kidney 293 (HEK-293) cell line, were eliminated from the bloodstream of experimental animals within much shorter periods of time (MRTs = 60-100 min).

Using the previously defined sets of recombinant human and bovine AChE molecular arrays (Kronman *et al.*, 2000; Chitlaru *et al.*, 2002) as well as a similar set of newly-cloned and expressed rhesus AChE, we now reveal that specific amino-acid domains promote the elimination of soluble primate AChEs from the circulation of rhesus monkeys but not of mice, thereby mitigating the positive contribution of post-translation-related processing to circulatory retention. This specific process for removal of homologous AChE molecules by macaques may however be countermanded by the effective masking of these domains, for example by chemical conjugation of polyethylene glycol (PEG) moieties, resulting in the generation of primate AChEs exhibiting extraordinarily long-term circulatory residence in monkeys.

## METHODS

### Cell culture techniques, enzyme production and purification of rAChEs

Generation of HEK-293 cell lines stably expressing high levels of rHuAChE, and rBoAChE was described previously (Kronman *et al.*, 2000; Chitlaru *et al.*, 2001). Generation of a HEK-293 cell line stably expressing high levels of rRhAChE was performed in the same manner, by transfecting the cells with the pRhAChE-nc vector (see below), followed by G418 selection to form rRhAChE stable producer cells. The generation of *in-vivo* highly sialylated rHuAChE (wild type and tetraglycosylated mutant), rBoAChE and rRhAChE was achieved by stably expressing the human (wild type or D61N mutant), bovine and rhesus acetylcholinesterase genes, in the genetically modified 293ST 2D6 cells that express high levels of heterologous 2,6-sialyltransferase followed by G418 selection to form rAChE stable producer cells, as described previously (Chitlaru *et al.*, 1998). The method for purification of the secreted rHuAChE and rBoAChE enzymes was described previously (Kronman *et al.*, 1995) and served also for the purification of secreted rRhAChE.

### DNA sequencing

The coding sequence of the rhesus AChE gene was determined through PCR amplification of rhesus genomic adult female lymphocyte DNA fragments. Five amplified overlapping DNA fragments were generated, using primers based on the human AChE sequence (see Fig. 14A). Sequence determination (ABI prism rhodamine kit using the ABI310 Genetic Analyzer, Applied Biosystems) was based on two independent clone isolates for each amplified DNA fragment. Sequences of exons 2 to 6 of the rhesus AChE gene were deposited in the Genebank database under accession numbers AY372522 to AY372526.

### Generation of the RhAChE cDNA

To generate the recombinant rhesus AChE gene, the human AChE gene cloned in the pBluescript vector (Stratagene, USA) was subjected to site-directed mutagenesis to replace human-specific codons with those coding for rhesus amino acids. Using the QuikChange site-directed mutagenesis kit (Stratagene, USA), each of the codons ATG, CAC and GCA, corresponding to amino acids Met42, His284 and Ala467 of the human AChE gene was replaced individually with the codons ACA, AAT and ACG, respectively, to code for Thr42, Asn284 and Thr467. The fragments *EcoRV-BstEII* containing the rhesus codon coding for Thr42, *BstEII-SbfI* containing the rhesus codon coding for Asn284, and *SbfI-NotI* containing the rhesus codon coding for Thr467, were excised from the corresponding mutated pBluescript vectors and used to replace the parallel fragments in the pAChE-nc expression vector (Kronman *et al.*, 1992), to generate the pRhAChE-nc vector. The AChE coding region within pRhAChE-nc was verified by DNA sequencing as described above.



### Enzyme activity

AChE activities of purified AChE preparations were measured according to Ellman *et al.* (1961). Assays were performed in the presence of 0.5 mM acetylthiocholine, 50 mM sodium phosphate buffer pH 8.0, 0.1 mg/ml BSA and 0.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid). The assay was carried out at 27°C and monitored by a Thermomax microplate reader (Molecular Devices).

To allow measurement of residual AChE activity in rhesus macaque serum samples, endogenous butyrylcholinesterase (BChE) background activity was reduced by pretreatment of the samples with the BChE-specific inhibitor, tetraisopropylpyrophosphoramidate (iso-OMPA). This was achieved by preincubating the serum samples in the presence of 50 mM sodium phosphate buffer pH 8.0/0.1 mg/ml BSA/ 0.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid) and  $4 \times 10^{-5}$  M iso-OMPA for 10 minutes at room temperature before adding the substrate, acetylthiocholine (final conc. = 0.5 mM). Under these conditions, >98% of the endogenous BChE activity was inhibited without affecting AChE activity.

### Removal of sialic acid from rAChE

Recombinant HuAChE, BoAChE and RhAChE (15-30 nmol enzyme) in PBS were incubated for 16h with 0.2-0.4U of agarose-bound sialidase at room temperature. Sialidase was removed by Eppendorf centrifugation. Desialylated enzyme was dialyzed against PBS to remove free sialic acid.

### *In-vitro* tetramerization of rAChE

Synthesis and quality control of human ColQ- PRAD peptide CLLTPPPPLFPPPFRRG were described previously (Kronman *et al.*, 2000). Preparative tetramerization for the generation of milligram amounts of tetrameric rHuAChE (wild type or D61N mutant), rBoAChE and rRhAChE for pharmacokinetic studies was performed by incubating 70 nmol of highly sialylated rAChE with 140 nmol PRAD synthetic peptide for 12-16h at room temperature, in the presence of 5mM phosphate buffer pH 8.0 in a final volume of 2ml. Prior to administration to monkeys, the various *in-vitro* tetramerized rAChEs were dialyzed extensively against PBS. Complete conversion of the enzymes into tetrameric forms was verified by sucrose-gradient density analysis, as described previously (Kronman *et al.*, 2000).

### MALDI-TOF analysis of glycans

N-glycans of rAChEs (~100 µg protein) were purified, 2-aminobenzamide labeled (Bigge *et al.*, 1995) and converted into their neutral methylated forms by methyl iodide esterification, as described previously (Kronman *et al.*, 2000). Mass spectra of 2-aminobenzamide-labeled esterified rAChE glycans were acquired on a Micromass ToFSpec 2E reflectron time-of-flight (TOF) mass spectrometer. Samples of 1 µl of glycan samples diluted 1:10 in water were mixed

with an equal volume of freshly prepared 2,5-dihydroxybenzoic acid (10mg/ml in 70% acetonitrile) and loaded onto the mass spectrometer target. Dried spots were recrystallized by adding 0.5  $\mu$ l ethanol and allowed to dry again. Glycans were observed as  $[M+Na]^+$  ions. 1  $\mu$ l of peptide mixture (renin substrate, ACTH fragment 18-39, and angiotensin, 10 pMole/ $\mu$ l all from Sigma), which served as a three-point external calibrant for mass assignment of the ions, was mixed with freshly prepared  $\alpha$ -cyano-4-hydroxycinnamic acid (10 mg/ml in 49.5% acetonitrile; 49.5% ethanol; 0.001% TFA), loaded on the mass spectrometer target and allowed to dry. All oligosaccharides were analyzed at 20 kV with a single-stage reflectron in the positive-ion mode. Between 100 and 200 scans were averaged for each of the spectra shown.

### Conjugation of polyethylene glycol to AChE

Attachment of PEG chains to primary amines in rHuAChE was performed using succinimidyl propionate activated methoxy PEG (SPA-PEG; Shearwater polymers, Inc.), as described previously (Laub and Gallo, 1996). Briefly, purified rHuAChE or rRhAChE (5 $\mu$ M) were incubated with PEG-20000 at a ratio of 50:1  $[PEG]_0/[AChE \text{ primary amines}]_0$  in 50mM phosphate buffer pH 8.0 for 2 hours at room temperature. The chemically modified products were dialyzed extensively against phosphate buffer saline (PBS) and analyzed on 6% SDS-PAGE gels.

### Animal procedures

Female rhesus macaques (*Macaca mulatta*, 2.7-3.0 kg) were obtained from Covance, USA. Animals were quarantined upon arrival and screened for evidence of disease. The animals were individually housed in stainless-steel cages in animal rooms that were maintained at 20-22°C and relative humidity of 50 $\pm$ 10% on a 12-hr light-dark cycle. The monkeys were fed commercial certified Primate Chow (Koffolk, Inc., Tel-Aviv, Israel) and provided with tap water *ad libitum*. Male outbred ICR mice (Charles River Laboratories, UK) were maintained at 20-22°C and relative humidity of 50 $\pm$ 10% on a 12-hr light-dark cycle, fed with commercial rodent chow (Koffolk, Inc., Tel-Aviv, Israel) and provided with tap water *ad libitum*. Treatment of animals was in accordance with regulations outlined in the USDA Animal Welfare Act and the conditions specified in *The Guide for Care and Use of Laboratory Animals* (National Institute of Health, 1996). All pharmacokinetic studies (see below) in monkeys and mice were approved by the local ethical committee on animal experiments.

### Clearance experiments and analysis of pharmacokinetic profiles in mice and rhesus macaques

Following extensive dialysis against PBS (pH 7.4), 1000 units of the various rAChEs (165  $\mu$ g and 330  $\mu$ g and 165  $\mu$ g for the human, bovine and rhesus forms, respectively) were injected i.v. to 3 rhesus macaques, (injection volumes <1ml/kg). Samples (0.25 ml) of blood were collected at various periods of time in Microtainer tubes (Becton, Dickinson and Co., USA), centrifuged for 1 minute at 10,000 rpm in an Eppendorf microfuge and stored at  $-20^{\circ}\text{C}$  until AChE activity in serum samples was determined. Enzymatic activity was determined following iso-OMPA-mediated BChE inhibition, as described above. AChE activity values in samples removed 2 minutes after injection were referred to as input activities and were used for the calculation of residual activity throughout the experiment. AChE values were corrected for background activity determined in blood samples withdrawn 1h before performing the experiment. Exogenously administered AChE was at least 20-fold higher than background endogenous iso-OMPA-resistant ChE activity. To avoid the possible generation of anti-ChE antibodies, which might affect the circulatory retention times of the injected proteins, in all cases each of the monkeys were administered only once with ChE.

Clearance experiments in mice (3 to 6 mice per enzyme sample) were carried out essentially as described previously (Kronman *et al.*, 1995). Mice were injected i.v. with the various rRhAChE preparations (100 units/mouse in 0.2 ml PBS). Residual AChE activity in blood samples was measured and all values were corrected for background hydrolytic activity in the blood (using samples withdrawn 1 hour before performing the experiment). AChE activity values in samples removed 1 minute after injection were assigned a value of 100% and used for calculation of residual activity. Background cholinesterase levels in blood of pre-administered mice were less than 2 units/ml.

The clearance patterns of the various enzyme preparations were usually biphasic and fitted to a bi-exponential elimination pharmacokinetic model ( $C_t = Ae^{-k\alpha t} + Be^{-k\beta t}$ ) as described previously (Kronman *et al.*, 2000, Chitlaru *et al.*, 2001). This model enables determination of the parameters A and B which represent the fractions of the material removed from the circulation in the first-fast and second-slow elimination phases respectively, and  $T_{1/2\alpha}$  and  $T_{1/2\beta}$  which represent the circulatory half-life values of the enzyme in the fast and slow phases. The pharmacokinetic parameter MRT (mean residence time, which reflects the average length of time the administered molecules are retained in the organism) was independently obtained by analyzing the clearance data according to a noncompartmental pharmacokinetic model using the WinNonlin computer program (Laub and Gallo, 1996).

**Clearance experiments of native and radiolabeled RhBChE in rhesus macaques**

Serum BChE collected from rhesus macaques was purified on procainamide columns as described previously (Kronman *et al.*, 1995). Purified native BChE was radiolabeled by incubating the enzyme (7.2 nmole) with a tritiated organophosphorus compound, [<sup>3</sup>H]-diisopropyl phosphorofluoridate (DFP) (60μCi, 8.4Ci/mmol; Amersham Bioscience). Excess radioactive material was removed by passing the labeling mixture on a G-50 column. The labeled enzyme was administered to 3 rhesus macaques, blood samples were removed at various time periods and residual labeled BChE in blood samples was measured by radioactivity counting of trichloroacetic acid (TCA) precipitates in a liquid scintillation counter. Radioactivity values in samples removed 2 minutes after injection were assigned a value of 100% and used for residual-enzyme calculation.

To determine the pharmacokinetic profile of non-labeled rhesus serum BChE, ~10,000U of the enzyme was administered to a single monkey. Residual BChE enzymatic activity in blood samples removed at various periods of time was determined as described above, using butyrylthiocholine as the substrate.

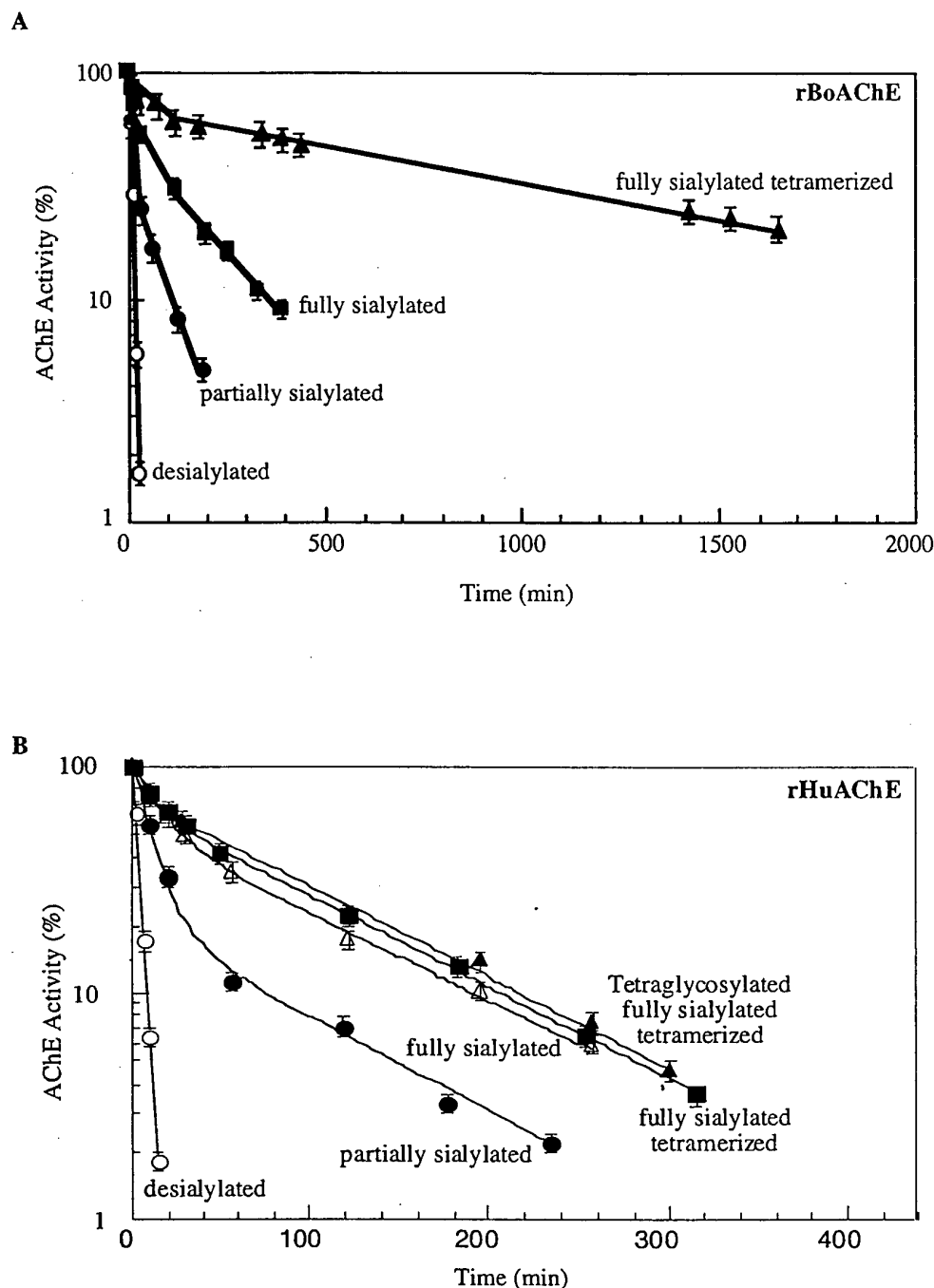
## RESULTS AND DISCUSSION

### Pharmacokinetics of recombinant BoAChE forms obey the same hierarchical rules in rhesus macaques and mice

The effect of post-translation processing on the circulatory retention of heterologous AChE in non-human primates was addressed by monitoring the pharmacokinetic profiles of an array of differently processed recombinant bovine AChEs in rhesus macaques. The contribution of sialic acid contents to the circulatory retention of the recombinant enzyme forms was evaluated by comparing the clearance profiles of enzymatically desialylated enzyme, partially sialylated enzyme produced by HEK-293 producer cells, and fully sialylated enzyme produced by the modified 293ST-2D6 cell line which coexpresses high levels of recombinant  $\alpha$ 2,6-sialyltransferase (Chitlaru *et al.*, 1998). The effect of enzyme subunit assembly on circulatory residence of the recombinant AChE was evaluated by examining the pharmacokinetic behavior of fully sialylated enzyme following its complete conversion into tetramers in the presence of the synthetic ColQ Proline Rich Association Domain (PRAD) peptide (Kronman *et al.*, 2000, Simon *et al.*, 1998).

Pharmacokinetic analyses of the various recombinant bovine AChE forms in rhesus macaques demonstrated that each of the differently processed forms was characterized by a unique circulatory retention pattern (Fig. 13A, Table 5). Recombinant bovine AChE totally devoid of sialic acid residues displayed a monophasic elimination curve and was cleared rapidly from the circulation within a very short period of time (Mean residence time (MRT) =  $3.9 \pm 0.6$  min). In contrast, all recombinant bovine enzyme forms containing sialic acid residues displayed biphasic elimination curves with different circulatory residence values in accordance with the level of sialic acid occupancy and enzyme subunit assembly. Thus, rBoAChE preparations consisting of partially sialylated, fully sialylated and fully sialylated/tetramerized forms of the enzyme exhibited increasingly higher circulatory retention rates, as demonstrated by the corresponding MRT values of  $66 \pm 2$ ,  $205 \pm 7$  and  $1510 \pm 45$  minutes, respectively. These results establish that both sialic acid levels and enzyme subunit assembly strongly influence circulatory retention of BoAChE in non-human primates and that enzyme sialylation and oligomerization operate in a synergistic manner, resulting in the generation of an enzyme form which resides in the circulation of rhesus macaques for very long periods of time.

The MRT values of the various rBoAChE forms in monkeys are very similar to those obtained in mice (Kronman *et al.*, 2000; See also Fig. 17). Thus, the ratios of the MRTs in rhesus to the MRTs in mice ( $MRT_{Rh}:MRT_M$ ) for the asialylated, partially-sialylated, fully-sialylated and fully-sialylated/tetramerized forms of rBoAChE are  $1.0 \pm 0.05$ ,  $1.2 \pm 0.09$ ,  $1.0 \pm 0.06$  and  $1.1 \pm 0.08$ , respectively. The finding that the  $MRT_{Rh}:MRT_M$  values for all the differently



**Fig. 13: Comparison of the circulatory elimination profiles of various molecular forms of rBoAChE and rHuAChE in rhesus macaques.** Purified preparations of various molecular forms of rBoAChE (*A*) or rHuAChE (*B*), differing in their post-translation processing (glycan sialylation level, subunit assembly state, N-glycan loading) were administered to 3 rhesus macaques and residual AChE activity in the circulation was assayed at the indicated time points. Circulatory removal curves were determined for all the AChE forms and the derived pharmacokinetic parameters are presented in Table 5. Note the difference in timescales in panels A and B.

processed forms of rBoAChE are approximately 1, demonstrates that the pharmacokinetics of rBoAChE is determined in the rhesus animal system by the same set of principles revealed in the past in mice (Kronman *et al.*, 2000), reflecting the hierarchical rules of fully-sialylated/tetramerized > fully-sialylated > partially-sialylated > asialylated.

**Table 5: Pharmacokinetic parameters of rBoAChE and rHuAChE in rhesus macaques**

AChE source	AChE type	A (%)	B (%)	$T_{1/2\alpha}$ (min)	$T_{1/2\beta}$ (min)	MRT (min)
rBoAChE	sialidase treated	100	-	3.5±0.5	-	3.9±0.6
	partially sialylated	85±6	20±5	5±0.5	57±4	66±2
	fully sialylated	56±6	46±5	17±4	163±25	205±7
	fully sialylated, tetramerized	34±2	68±2	14±2	1080±30	1510±45
rHuAChE	sialidase treated	100	-	2±0.5	-	2.7±0.4
	partially sialylated	85±5	17±4	6±1	54±6	59±1
	fully sialylated	37±4	65±6	6±2	73±4	99±3
	fully sialylated, tetramerized	31±4	72±5	5±1	74±4	96±5
	Tetraglycosylated, fully sialylated, tetramerized	40±3	64±4	6±1.5	73±5	97±6

**The circulatory elimination patterns of recombinant human AChEs in rhesus macaques appear to deviate from the classical hierarchical rules established in mice**

The generation of a therapeutically effective bioscavenger of organophosphate compounds based on exogenously administered cholinesterase, would ideally require the use of an enzyme form of homologous or closely related origin. We therefore addressed the question, whether the set of rules governing bovine AChE pharmacokinetics in monkeys apply to primate enzyme species as well. To this end, we monitored the circulatory performance of human AChE in rhesus macaques. Pharmacokinetic analyses of differently processed forms of human rAChE in

monkeys revealed a unique pattern of elimination profiles (Fig. 13B, Table 5). Human rAChE entirely devoid of sialic acid residues was eliminated rapidly from the circulation ( $MRT = 2.7 \pm 0.4$  min) and partial sialylation of the rHuAChE resulted in improved circulatory residence ( $MRT = 59 \pm 1$  min), yet, full sialylation of the human enzyme resulted in an additional 1.7-fold increase in circulatory retention only ( $MRT = 99 \pm 3$  min), while tetramerization of the fully sialylated human enzyme did not further contribute to the circulatory retention of the human enzyme ( $MRT = 96 \pm 5$  min). Thus, while to full sialylation of the bovine enzyme and its following conversion into tetramers resulted in a 2.8-fold and 7-fold increase in circulatory residence time, respectively, full sialylation of the human enzyme form resulted in only a modest increase in MRT, and its subsequent tetramerization did not affect its circulatory retention at all. Notably, when examined in mice (Chitlaru *et al.*, 2001, see also Fig. 17B), the circulatory residence of the fully sialylated human enzyme could be significantly extended by tetramerization ( $MRT_{\text{fully-sialylated}}$  and  $MRT_{\text{fully-sialylated/tetrameric}} = 195 \pm 9$  and  $740 \pm 30$  min, respectively) These results indicate that the inability to significantly extend the circulatory lifetime of rHuAChE by tetramerization in rhesus macaques does not reflect an intrinsic property of the human enzyme *per se*, but rather some property of the rhesus macaque animal system with regard to the enzyme form of human source.

Human rAChE differs from the bovine enzyme in its number of appended N-glycans. The human enzyme contains three N-glycans at amino acid positions, which are homologous to those of three N-glycans of the bovine enzyme, while the latter carries an additional fourth N-glycan at amino acid 61 (Mendelson *et al.*, 1998; Velan *et al.*, 1993). Previous findings showed that a mutated form of fully sialylated, tetramerized rHuAChE, which contains, like the bovine enzyme, a fourth N-glycan at amino acid 61, resides in the circulation of mice for longer periods of time than its triglycosylated counterpart (Chitlaru *et al.*, 2002, see also Fig. 17B). To examine whether increasing the number of N-glycans contributes to the retention of rHuAChE in the rhesus macaque background as well, the pharmacokinetic behavior of the tetraglycosylated D61N enzyme in its fully sialylated/tetramerized configuration was monitored in monkeys. Pharmacokinetic profiling of this enzyme form demonstrated that its residence was virtually identical to that of its triglycosylated counterpart ( $MRT$  values =  $97 \pm 6$  and  $96 \pm 5$  min, respectively, Table 5 and Fig. 17B), and thus, unlike in the mouse animal system, where both enzyme tetramerization and addition of N-glycans, increased the circulatory retention of human AChE, in the rhesus animal system, neither tetramerization nor addition of N-glycans, contributed in a measurable manner to the circulatory residence of the fully sialylated enzyme.

These findings suggest that rHuAChE pharmacokinetics in the rhesus animal system deviate from the classical hierarchical rules found for the same enzyme in mice due to the operation of a process which specifically promotes elimination of the human enzyme from the circulation of monkeys, while mitigating the positive effect of post-translation-related processes (i.e. enzyme assembly and glycan loading) on circulatory retention. This inability to augment circulatory retention of AChE in monkeys cannot be attributed to quantitative or qualitative differences in

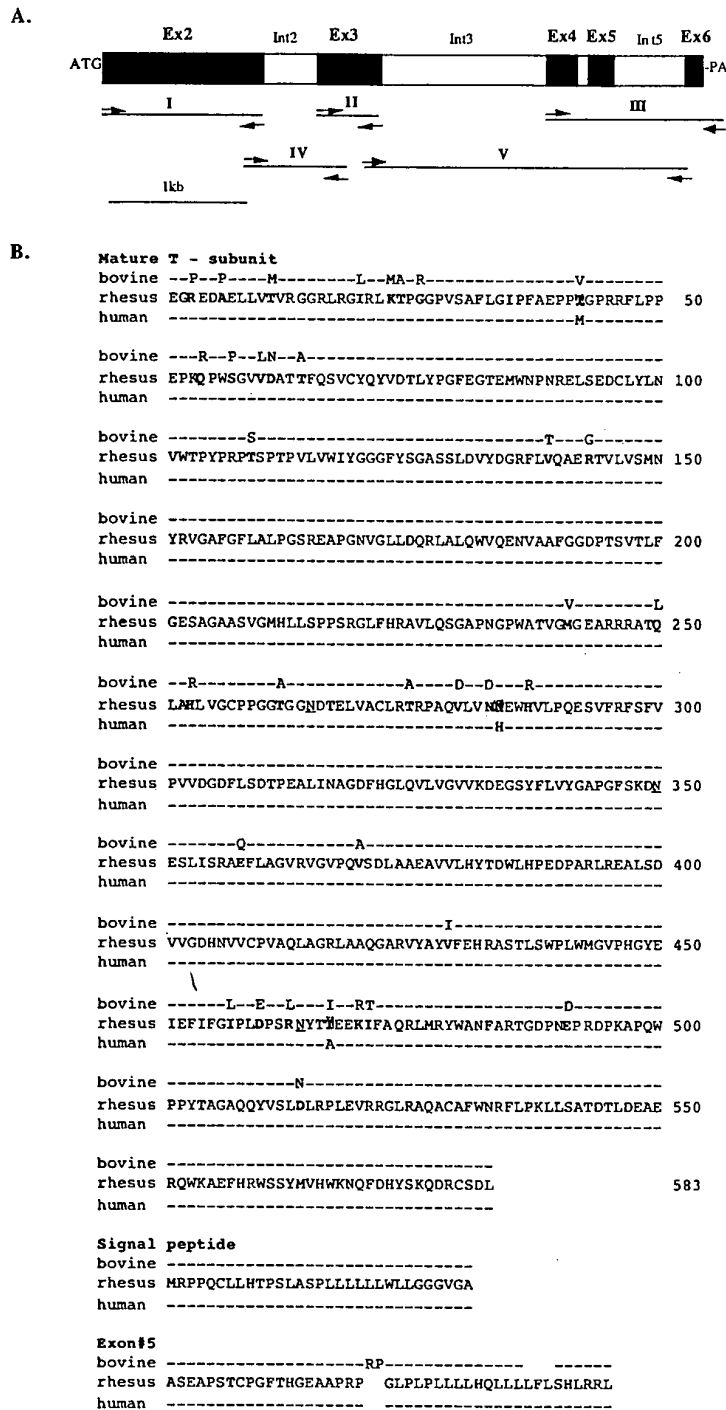


glycan processing of the two enzymes, since: (a) the tetraglycosylated mutant form of rHuAChE possesses the same number of appended N-glycans at the same amino acid positions as the bovine enzyme (Mendelson *et al.*, 1998), and (b) extensive structural analyses carried out in the past (Kronman *et al.*, 2000; Chitlaru *et al.*, 2001; see also Fig. 15B) have clearly demonstrated that both the bovine and human versions of rAChE generated in the HEK-293 cell line contain almost identical N-glycan forms in similar abundances. Thus, the differential pharmacokinetic hierarchical patterns exhibited by human and bovine AChEs in the rhesus animal system cannot be attributed to points of variance in their post-translation modifications, but most likely stem from differences in their primary amino acid sequences.

### **Cloning, expression and N-glycan analysis of various posttranslationally modified recombinant RhAChE forms**

The presence of butyrylcholinesterase (BChE) and not acetylcholinesterase in the circulation of primates (e.g. rhesus and human) at the adult stage (Li *et al.*, 2000), may suggest that AChE is actively removed from the bloodstream. Such a mechanism could provide an explanation for the unique elimination of human AChE from the circulation of monkeys, since unlike bovine AChE, this species of the enzyme may be sufficiently similar to the "self" AChE of rhesus macaques and therefore be subject to active removal. To examine whether the pharmacokinetic patterns of human AChE enzyme forms may indeed be attributed to the action of "self" AChE circulatory elimination mechanisms, we set out to examine the pharmacokinetics of rhesus AChE in its homologous animal system. Determination of the genomic sequences of rhesus macaque AChE corresponding to exons 2 to 6 (Fig. 14A) revealed that at the DNA level, the rhesus and human AChEs differ at 32 out of 1971 nucleotides. Only three of these divergences result in changes in amino acids within the coding region of the mature enzyme: M42<sub>human</sub>->T42<sub>rhesus</sub>, H284<sub>human</sub>->N284<sub>rhesus</sub> and A467<sub>human</sub>->T467<sub>rhesus</sub> (Fig. 14B). The rRhAChE coding sequences were introduced into HEK-293 cells, or the modified 293ST-2D6 cell line expressing high levels of recombinant  $\alpha$ -2,6 sialyltransferase (Chitlaru *et al.*, 1998), to generate authentic rhesus macaque recombinant AChEs, which are partially or fully sialylated, respectively.

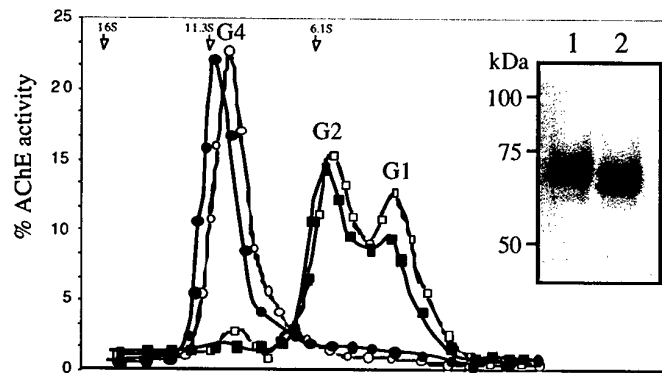
As expected, the levels of production of the partially and fully sialylated rRhAChEs were similar to those of the human enzyme expressed in the same cell systems (approx. 50 units/ml/24h, (Chitlaru *et al.*, 1998)). The rRhAChE products exhibited a migration pattern on SDS-PAGE (Fig. 15A, inset), indistinguishable from that of their human counterparts (Chitlaru *et al.*, 1998). As shown previously for the human enzyme, the rhesus AChE derived from the modified 293ST-2D6 cell line displayed a slower mobility, reflecting the efficient sialylation of the enzyme product in these  $\alpha$ -2,6 sialyltransferase expresser cells. This was further verified by the pharmacokinetic analysis of the enzyme product in mice, as described below.



**Fig. 14: Determination of rhesus AChE gene sequence (A)** Map of the *Macaca mulatta* AChE gene and DNA genomic fragments generated by PCR amplification for sequence determination. **(B)** Amino acid sequence of RhAChE. The deduced sequence of the mature T-subunit, signal peptide and the C-terminus of the H subunit (Exon 5) of RhAChE, and the corresponding regions of HuAChE and BoAChE are shown. The conserved N-glycosylation consensus sequences are underlined. The rhesus/bovine and rhesus/human diverging amino acids are highlighted in bold and shadowed fonts, respectively.

Examination of the sucrose-gradient profile of partially or fully sialylated rRhAChE demonstrated that these enzyme preparations consist of a mixture of differently assembled forms, most of which are dimers. Incubation of these rRhAChE preparations with the synthetic human ColQ PRAD peptide (Kronman *et al.*, 2000) resulted in the quantitative conversion of the enzymes into assembled tetramers (Fig. 15A), in manner similar to that reported previously for human AChE (Chitlaru *et al.*, 2001).

To determine that the basic structures of the N-glycans appended to rRhAChE generated in HEK-293 cells are similar to those of rBoAChE and rHuAChE derived from the same cells, the glycan structures of rRhAChE (partially and fully sialylated) were analyzed by MALDI-TOF (Fig 15B). Inspection of the MALDI-TOF-MS profiles of desialylated glycans of rRhAChE produced by HEK-293 cells and sialyltransferase-modified 293ST-2D6 cells revealed that both glycan pools consist of a similar array of varied structures, displaying an overall pattern which is similar to that of the N-glycans associated with partially sialylated and fully sialylated rHuAChE and rBoAChE (Fig.15B). Thus, the major glycan species (41-46% of total glycans) correspond to complex-type biantennary forms carrying a fucose moiety, while triantennary glycans (22-30% of the total glycans) were represented to a higher extent by the non-fucosylated form. The single significant difference between the glycan structures of rRhAChE and those of rHuAChE and rBoAChE is in the level of tetraglycosylated forms, which is higher in the rhesus enzymes. Taken together, characterization of the recombinant RhAChE demonstrates that this enzyme displays a strikingly high degree of similarity to the human enzyme both in its primary sequence and in its post-translation processing.

**A****B**

biantennary		triantennary		tetraantennary	
rAChE:	rAChE-ST:	rAChE:	rAChE-ST:	rAChE:	rAChE-ST:
Rh: 5.8±1	3.9±0.5	Rh: 20±2	17±1.8	Rh: 11±1.5	19±2
Hu: 4.9±0.8	5.2±0.6	Hu: 13.6±1	19±1	Hu: <0.8	<0.8
Bo: 6.9±1	5.9±0.7	Bo: 25±2	23±2	Bo: <0.8	<0.8

biantennary fucosylated		triantennary fucosylated		tetraantennary fucosylated	
rAChE:	rAChE-ST:	rAChE:	rAChE-ST:	rAChE:	rAChE-ST:
Rh: 41±4	46±5	Rh: 10±1.8	5.5±0.8	Rh: 5.8±1	9±1
Hu: 65±6	61±5	Hu: 11.2±2	14.5±2	Hu: 2.2±0.3	2.8±0.8
Bo: 57±5	54±5	Bo: 5.9±2	8±2	Bo: 1.5±0.4	3.5±0.6

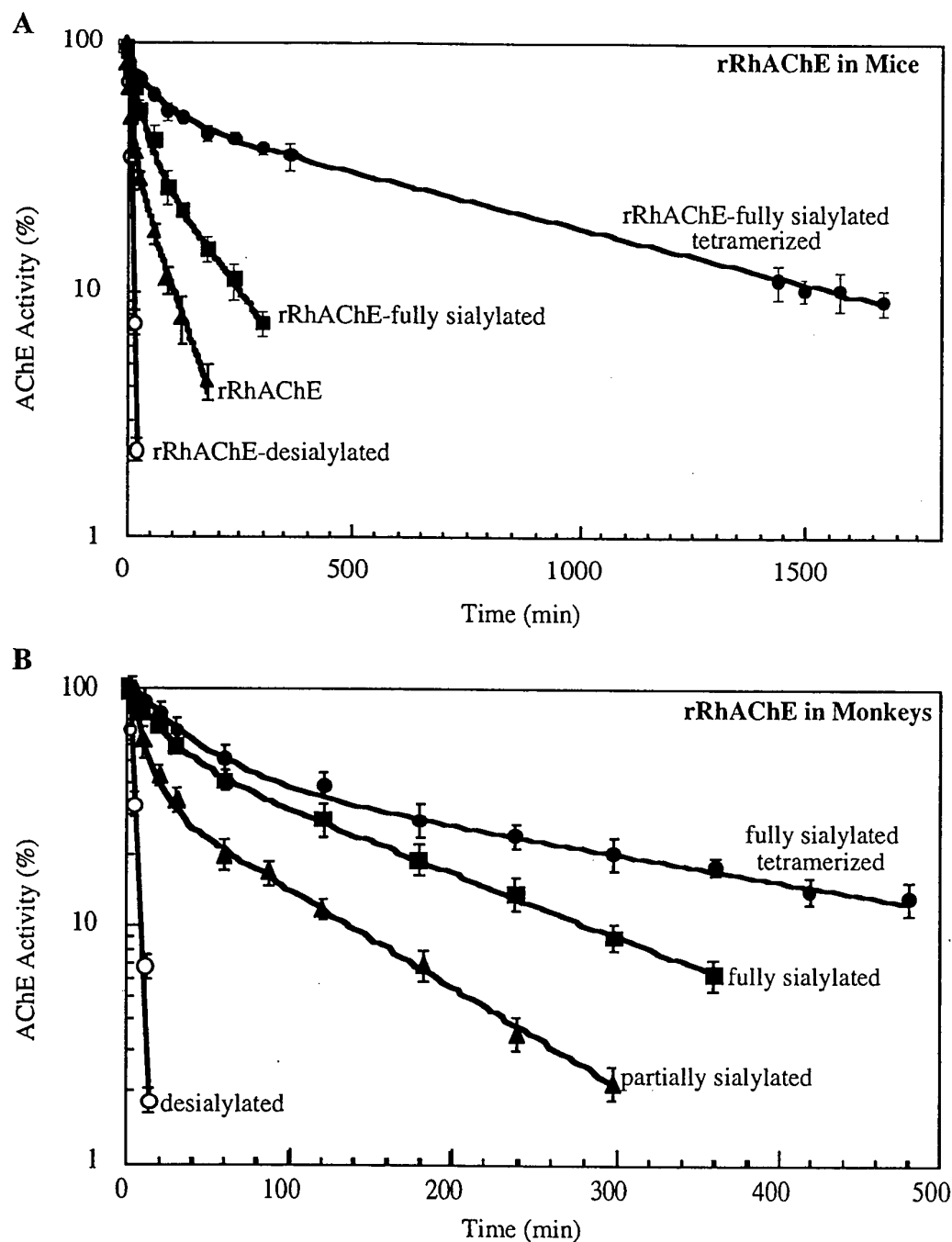
**Fig. 15: Characterization of recombinant rhesus AChE** (A) Sucrose gradient sedimentation profiles of partially sialylated (filled symbols) and fully sialylated (empty symbols) rRhAChE respectively, before (squares) and after (circles) *in vitro* tetramerization. G1, G2, and G4 annotate the monomeric, dimeric, and tetrameric RhAChE forms, respectively. Arrows denote the elution position of the sedimentation markers alkaline phosphatase (6.1 S), catalase (11.3 S), and  $\beta$ -galactosidase (16 S) included in all samples. **Inset:** SDS-PAGE analysis of rRhAChE produced in the sialyltransferase-expresser 293ST-2D6 cells (lane 1) or in non-modified HEK-293 cells (lane 2). (B) The basic structures of the glycans of rAChEs (produced by HEK-293 cells) and rAChE-STs (produced by 293ST-2D6 cells). Glycans are classified according to their branching and fucosylation. Desialylated glycan structures were deduced from the mass spectral data obtained by MALDI-TOF as described in the "Method" section. The relative abundance (% of total glycans) of each glycan species is shown and compared to the abundances of the corresponding glycan structures in rHuAChE and rBoAChE determined previously (Kronman *et al.*, 2000; Chitlaru *et al.*, 2001). Open square, GlcNAc; open circle, Mannose; solid circle,  $\beta$ -Galactose; solid square, GalNAc; elongated diamond, Fucose. Rh, rhesus; Hu, human; Bo, Bovine.

### Pharmacokinetic profiles of recombinant RhAChE in Rhesus macaques and mice

Examination of the pharmacokinetic behavior of the various forms of rRhAChE in mice (Fig. 16A, Table 6), demonstrated that rRhAChE is retained in the circulation in a manner similar to that displayed by recombinant human AChE (Kronman *et al.*, 2000). This was manifested both by the increase in circulatory residence time observed for enzyme forms displaying increasing amounts of appended sialic acid residues (MRT for asialylated, partially sialylated, and fully sialylated rRhAChE =  $3.2 \pm 0.6$ ,  $92 \pm 4$  and  $171 \pm 8$  min, respectively), and by the significant combined effect of both full sialylation and enzyme tetramerization (MRT of fully sialylated tetrameric rRhAChE =  $805 \pm 25$  min). Thus, rRhAChE pharmacokinetics in mice is governed, like rHuAChE, by post-translation-related processes that operate in a hierarchical manner leading to the extension of the circulatory residence by efficient sialylation and tetramerization. The strikingly similar pharmacokinetic behaviors of human and rhesus AChEs in the mice animal system and the fact that they are equally influenced by the same set of post-translation processes, is in accordance with the high degree of resemblance of the two primate AChE enzyme forms.

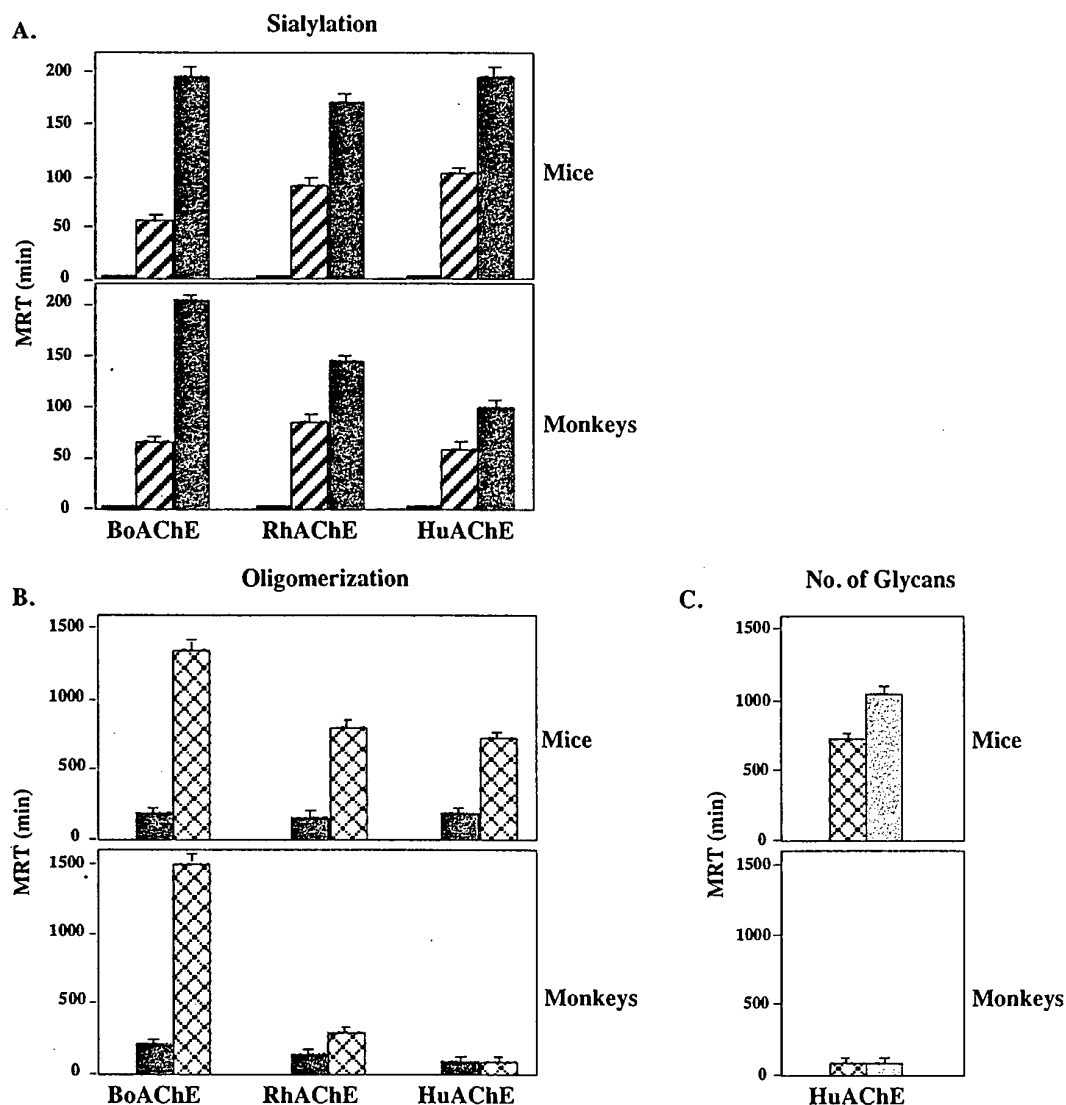
**Table 6: Pharmacokinetic parameters of rRhAChE in rhesus macaques and mice**

Animal system	AChE type	A (%)	B (%)	T1/2 $\alpha$ (min)	T1/2 $\beta$ (min)	MRT (min)
Rhesus	sialidase treated	100	-	$3.6 \pm 0.6$	-	$3.4 \pm 0.4$
	partially sialylated	$75 \pm 5$	$32 \pm 5$	$7 \pm 1$	$72 \pm 10$	$86 \pm 6$
	fully sialylated	$46 \pm 4$	$57 \pm 5$	$16 \pm 2$	$113 \pm 8$	$143 \pm 7$
	fully sialylated, tetramerized	$57 \pm 3$	$46 \pm 3$	$26 \pm 3$	$255 \pm 24$	$290 \pm 25$
Mouse	sialidase treated	100	-	$2.9 \pm 0.6$	-	$3.2 \pm 0.6$
	partially sialylated	$56 \pm 8$	$34 \pm 9$	$8 \pm 3$	$71 \pm 16$	$92 \pm 4$
	fully sialylated	$51 \pm 7$	$49 \pm 7$	$11 \pm 3$	$118 \pm 12$	$171 \pm 8$
	fully sialylated, tetramerized	$46 \pm 3$	$52 \pm 3$	$45 \pm 10$	$666 \pm 38$	$805 \pm 25$



**Fig. 16: Circulatory elimination profiles of various molecular forms of rRhAChE in mice and in rhesus macaques** Purified preparations of various molecular forms of rRhAChE, differing in their post-translation processing (glycan sialylation level, subunit assembly state), were administered to mice (**A**) or rhesus macaques (**B**) and residual AChE activity in the circulation was assayed at the indicated time points. Circulatory removal curves were determined for all the AChE forms and the derived pharmacokinetic parameters are presented in Table 6. Note the difference in timescales in panels A and B.

Pharmacokinetic analyses of the various recombinant rhesus AChE forms in monkeys demonstrated (Fig. 16B) that rhesus AChE pharmacokinetics abides by the classical rules of hierarchy to a lesser extent in this animal system. This is manifested by the finding that conversion of rRhAChE to its fully tetramerized state resulted in a 2-fold increase (MRT rRhAChE fully-sialylated =  $143 \pm 7$  min; MRT rRhAChE fully-sialylated/tetramerized =  $290 \pm 25$  min) in circulatory retention only, as opposed to a 4.7-fold increase in mice (MRT rRhAChE fully-sialylated =  $171 \pm 8$  min; MRT rRhAChE fully-sialylated/tetramerized =  $805 \pm 25$  min). Thus, while the  $MRT_{Rh}:MRT_M$  values of asialylated, partially-sialylated, and fully-sialylated rRhAChE are  $1.0 \pm 0.06$ ,  $0.93 \pm 0.04$ , and  $0.84 \pm 0.04$ , respectively, the  $MRT_{Rh}:MRT_M$  value of the fully-sialylated/tetramerized form of rRhAChE sharply declines to  $0.36 \pm 0.02$ . Thus, unlike bovine AChE which displayed a similar pharmacokinetic behavior in both mice and monkeys as exemplified by  $MRT_{Rh}:MRT_M$  values of approximately 1 for all the differently processed forms of rBoAChE, the rhesus enzyme resembles its human counterpart in its differential pharmacokinetic behavior in the mouse and monkey animal systems; circulatory retention of both primate enzymes was clearly less susceptible to post-translation modifications in monkeys. Taken together these findings demonstrate that the circulatory residence of bovine and primate AChEs in rhesus macaques are governed in a different manner. Increasing sialic acid levels of the different enzyme species results in extension of their circulatory lifetime in rhesus macaques (Fig. 17A), emphasizing the pivotal role of glycan sialylation in determining the circulatory residence of AChEs in rhesus macaques. Yet, one can notice that the effect of full sialylation on AChE pharmacokinetics in rhesus macaques is slightly less prominent in the case of the primate enzymes. The differential pharmacokinetic performance of bovine and primate AChEs in rhesus macaques is fully manifested when examining the effect of enzyme tetramerization on circulatory residence (Fig. 17B). While assembly of the bovine enzyme into tetramers resulted in a striking increase in its retention, tetramerization affected the circulatory duration of the primate AChEs to a much lower extent, or not at all. The deviation from the classical hierarchical rules established for the different enzyme species in the mice animal system, is further manifested by the inability to extend circulatory residence of the human enzyme by increasing the glycan loading of the enzyme (Fig. 17C). Thus, unlike the bovine enzyme, AChEs of primate source appear to be actively eliminated from the circulation of monkeys in a manner that counteracts the positive effect of post-translation processing on circulatory longevity. The somewhat surprising observation that the human enzyme is even more prone than the rhesus enzyme to elimination from the circulation of rhesus macaques suggests that the differential pharmacokinetics of bovine and primate AChEs in monkeys cannot be fully explained in terms of "self"-specific enzyme removal. Specific structural features which are common to the primate enzymes under examination, and not to the bovine enzyme, seem to facilitate the active removal of AChEs in rhesus macaques, while the observed differences



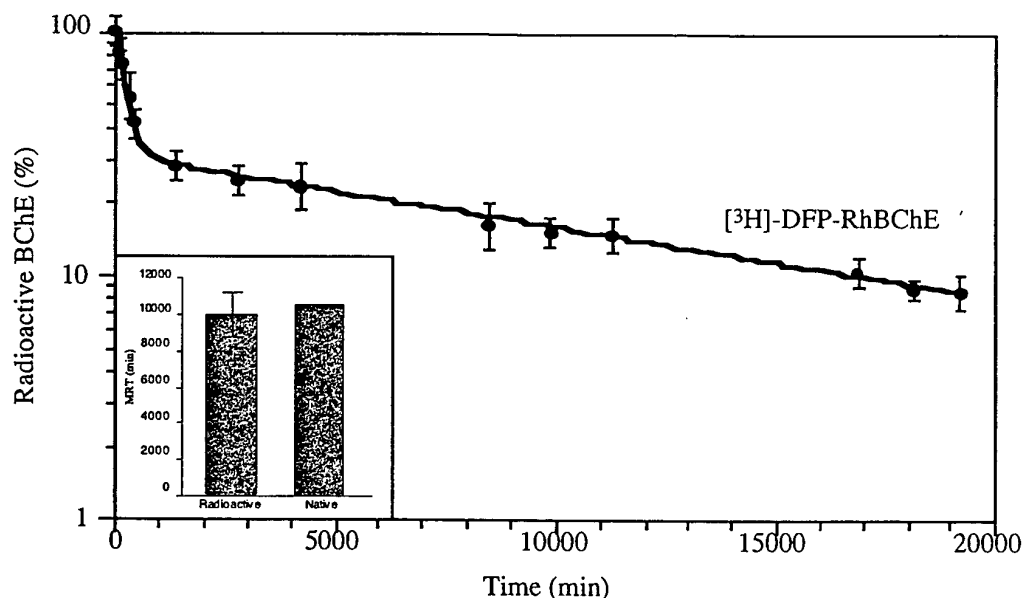
**Fig. 17: Effect of sialylation, oligomerization and glycan loading on the circulatory residence of bovine, human and rhesus AChEs in mice or rhesus macaques** Bars represent the MRT values in mice and monkeys of the different molecular forms of BoAChE, HuAChE and RhAChE as calculated from the circulatory elimination data presented in Figures 12 and 15 and in previous studies (Kronman *et al.*, 2000; Chitlaru *et al.*, 2001, 2002). (A) Effect of sialylation of AChEs: sialidase treated (■), partially sialylated (▧) and fully sialylated (▨). (B) Effect of oligomerization of sialylated AChEs: dimeric (■) and tetrameric (▩). (C) Effect of number of glycans in fully sialylated tetrameric HuAChEs: Wild type (wt) triglycosylated (▧) and D61N tetraglycosylated (▨).



in the clearance patterns of the human and rhesus enzymes in rhesus macaques may be attributed to subtle differences in structures of the two primate AChEs species.

**Facilitated elimination of cholinesterases from the circulation of rhesus macaques is restricted to primate AChE but not to primate BChE**

The presence of significant levels of circulating BChE in rhesus macaques (Carmona *et al.*, 1996) seems to suggest that active removal of primate cholinesterase in this animal system is restricted to AChE. However, primate BChE may also be actively and effectively eliminated from the circulation at rates similar to those of AChE, while its apparent prevalence in the circulation may be a reflection of its high turnover rate. To examine this issue, BChE from rhesus monkey serum was purified on procainamide columns and subjected to pharmacokinetic profiling in rhesus macaques. To enable the monitoring of the administered enzyme over the high background of endogenous BChE (~10U/ml), purified rhesus serum BChE was radiolabeled by incubating the enzyme with a tritiated organophosphorus compound, [<sup>3</sup>H]-DFP. The inactivated enzyme was then administered to rhesus macaques, and its circulatory residence was followed by determining radioactivity in blood samples removed at various periods of time (Fig. 18). This quantitation, which enables detection of relatively small amounts of administered enzyme was repeated in three monkeys, and allowed us to determine that rhesus BChE resides in the circulation of rhesus macaques for long periods of time, displaying an MRT value of  $9,950 \pm 1300$  min. However, to rule out the possibility that the coupling of the organophosphorus DFP compound to the enzyme affected significantly its circulatory retention, the experiment was performed with native non-labeled rhesus serum BChE, using large enough amounts of enzyme to allow significant quantitation of the administered enzyme over background, by monitoring BChE enzymatic activity. As in the case of the radiolabeled BChE, the native non-labeled form of rhesus BChE resided in the circulation for extended periods of time and displayed a similar MRT value of  $10,600 \pm 1200$  min (Fig. 18, inset). These values, which testify to the fact that RhBChE resides in the circulation of monkeys for very long periods of time, are in good agreement with those reported recently (Rosenberg *et al.*, 2002), and are similar to those determined in the past for human BChE in humans (Cascio *et al.*, 1988; Jenkins *et al.*, 1967). The occurrence of BChE in the circulation of rhesus macaques reflects therefore the long-term circulatory retention of this enzyme form, rather than the presence at steady state of high levels of short-lived species of ChE. These findings show that removal of rhesus AChE cannot be attributed to a general mechanism responsible for the elimination of all forms of primate cholinesterase, but rather depends on the operation of an elaborate clearance system, which recognizes and removes the soluble self acetylcholinesterase, RhAChE, and the very closely related HuAChE, in a unique manner.



**Fig. 18: Circulatory elimination profiles of labeled and non-labeled rhesus butyryl (BChE) in rhesus macaques.** Native rhesus BChE, labeled with [ $^3\text{H}$ ]-DFP was administered to 3 rhesus macaques and residual circulatory radiolabeled BChE was assayed at the indicated time points post-administration. **Inset:** Comparison of the MRT values of the radioactively-labeled rhesus BChE and enzymatically active non-labeled BChE, see "Methods" section.

#### **A possible role for amino acid domains in the differential pharmacokinetic behavior of bovine and primate AChEs in rhesus macaques**

As reported above, the glycan pool of rRhAChE consists of an array of varied structures whose basic structures displayed an overall pattern, similar to that of the N-glycans associated with rBoAChE and rHuAChE (Fig. 15B), the only notable difference being the relatively high level of tetraantennary glycan forms which is observed in the rhesus enzyme. Differences in the basic structures of appended N-glycans cannot therefore provide an explanation for the differential pharmacokinetic performances of bovine versus primate AChEs. Likewise, the dissimilar pharmacokinetic patterns of bovine and primate AChEs in monkeys cannot be attributed to differences in terminal sialylation, since the various AChEs are all efficiently sialylated in a similar manner when expressed in the genetically modified 293ST 2D6 cells that express high levels of heterologous 2,6-sialyltransferase (Fig. 15A, inset, see also (Chitlaru *et al.*, 1998)). Examination of the amino acid divergences between the human and rhesus AChEs (Fig. 14) demonstrates that these are not involved in the generation or abolishment of N-glycosylation consensus sequences, while SDS-PAGE analysis (Fig. 15A inset, and Chitlaru *et al.*, 1998) suggests that the human and rhesus enzymes carry similar amounts of glycans. Thus, both

primate AChEs carry 3 N-glycans per enzyme subunit, while the bovine enzyme contains 4 N-glycans per enzyme subunit. However, this quantitative difference in N-glycan loading of bovine AChE and primate AChEs is also apparently not responsible for the less efficient retention of the latter in the circulation of monkeys, since addition of a fourth N-glycan to the human enzyme at aa 61, the position which is analogous to that of the fourth N-glycan of the bovine enzyme, did not alter human AChE circulatory residence in monkeys (Fig. 17C). Furthermore, incubation of both primate and bovine forms of AChE with the synthetic ColQ PRAD peptide resulted in the quantitative conversion of all the various enzyme forms into tetramers in a similarly highly efficient manner, attesting to the fact that their disparate pharmacokinetics in monkeys are not related to differences in subunit organization.

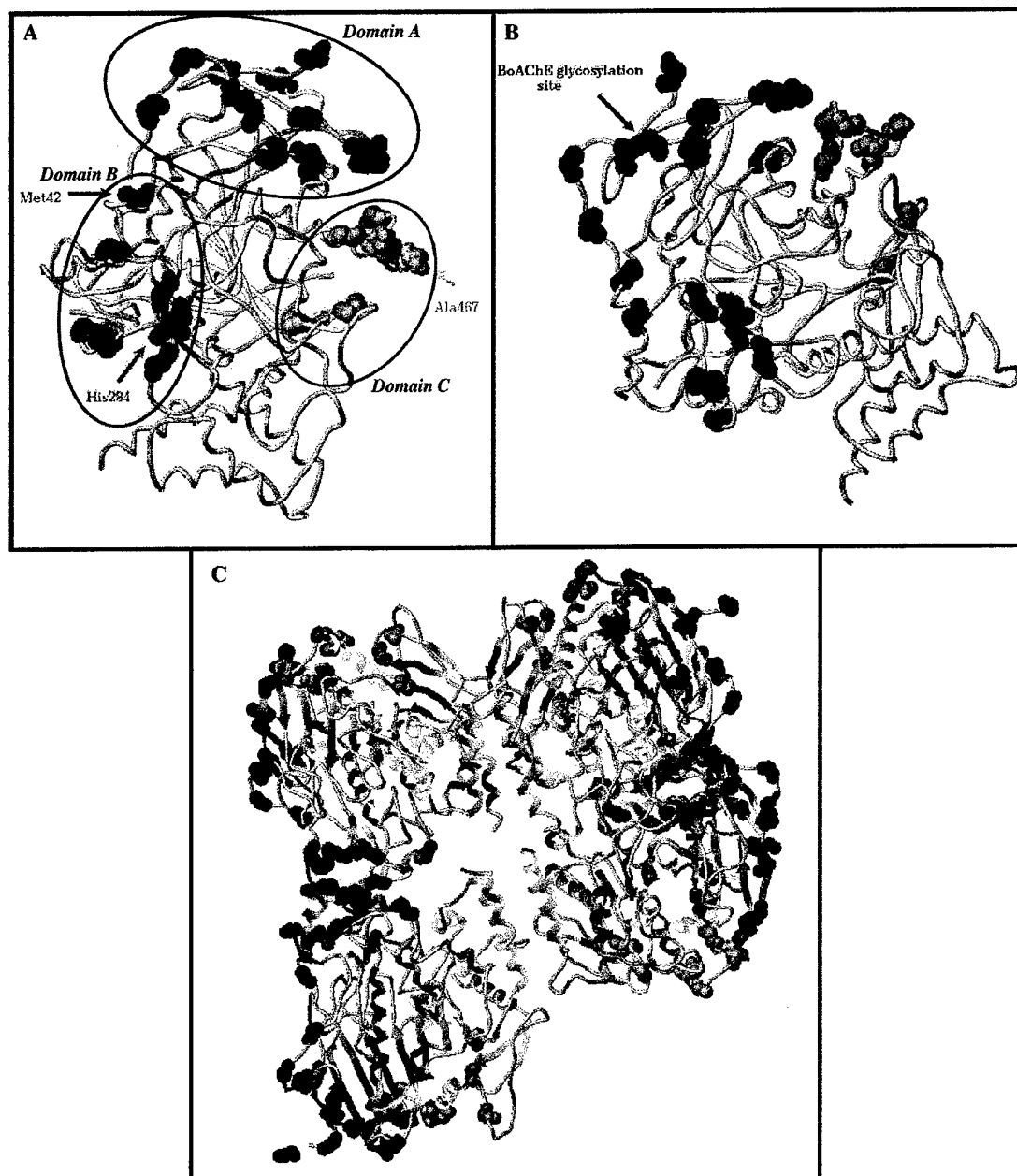
Taken together, all these findings suggest that the dissimilarity between bovine and primate AChE circulatory residence does not stem from differences in glycosylation or assembly state, but rather from points of divergence in their primary structure which differentially affect circulatory residence. The divergent amino acid domains may in themselves constitute ligands for receptor-directed clearance or can lead to appendage of yet undefined structures involved in clearance. Such species-specific amino acid domains which facilitate circulatory elimination of primate AChEs from the circulation of monkeys, operate through a mechanism of considerable potency as judged by their ability to counteract the positive effect of post-translation modifications on circulatory residence of primate AChEs, and function as clearance epitopes in an enzyme assembly-independent manner, as demonstrated by their ability to abolish tetramerization-induced circulatory life-time extension (Fig. 17B).

Alterations in plasma clearance of proteins by amino-acid replacements that affect interactions of the protein with its clearance receptor in a direct or indirect manner have been documented previously in other systems. In the case of insulin-like growth factor (IGF-I), the single and double amino acid mutants F49A and E3A/F49A were cleared more rapidly from the circulation due to their diminished ability to form high-molecular mass soluble complexes with insulin-like growth factor binding proteins, without changing the affinity of the mutant forms towards the Insulin growth factor type I receptor (Dubaque *et al.*, 2001). On the other hand, apolipoprotein E plasma clearance is severely compromised by a single Arg-158->Cys mutation, which prevents efficient binding of ApoE to the low-density lipoprotein receptor (van Vlijmen *et al.*, 1996). Amino acid substitutions were also demonstrated to affect tissue plasminogen activator (t-PA) clearance from the circulation, yet in this case the altered pharmacokinetic properties were due to changes in the N-glycan contents of the protein, rather than to the presence of altered amino acids *per se*. Thus, the 9-fold reduction in circulatory clearance of the Thr-110->Asn t-PA mutant is probably due to the addition of a complex-type N-glycan structure (Keyt *et al.*, 1994), while the 6.5-fold reduction in plasma clearance of the Asn117->Gln t-PA mutant was attributed to the prevention of high-mannose glycosylation at this site (Aoki *et al.*, 2001). As noted above, in the case of AChE the differential circulatory behavior of primate and bovine enzymes cannot be attributed to glycosylation-related factors.

Comparison of the sequences of the bovine and rhesus enzymes (Fig. 14B) demonstrates that the diverging amino acids are dispersed throughout the primary enzyme structures, yet, examination of the 3D structure of AChE reveals that all of these diverging amino acids are clustered within three domains, A, B and C (Fig. 19). These divergence patches are located at the surface of the enzyme, both in its monomeric (Fig. 19A) and tetrameric (Fig. 19C) states of assembly, only part of Domain B being obscured by tetramerization (Fig. 19C). The bovine version of AChE as well as the tetraglycosylated form of the human enzyme mentioned above carry an additional N-glycan at aa 61, which maps to a central position within Domain A (Fig. 19B). Interestingly, human AChE carrying this additional N-glycan, displayed enhanced circulatory retention in mice (Chitlaru *et al.*, 2002). We note that the three human/rhesus diverging amino acids, 42, 284 and 467, which are apparently responsible for the somewhat differential pharmacokinetic patterns of these two enzymes in the rhesus animal system, indeed map within Domains B (42 and 284) and C (467).

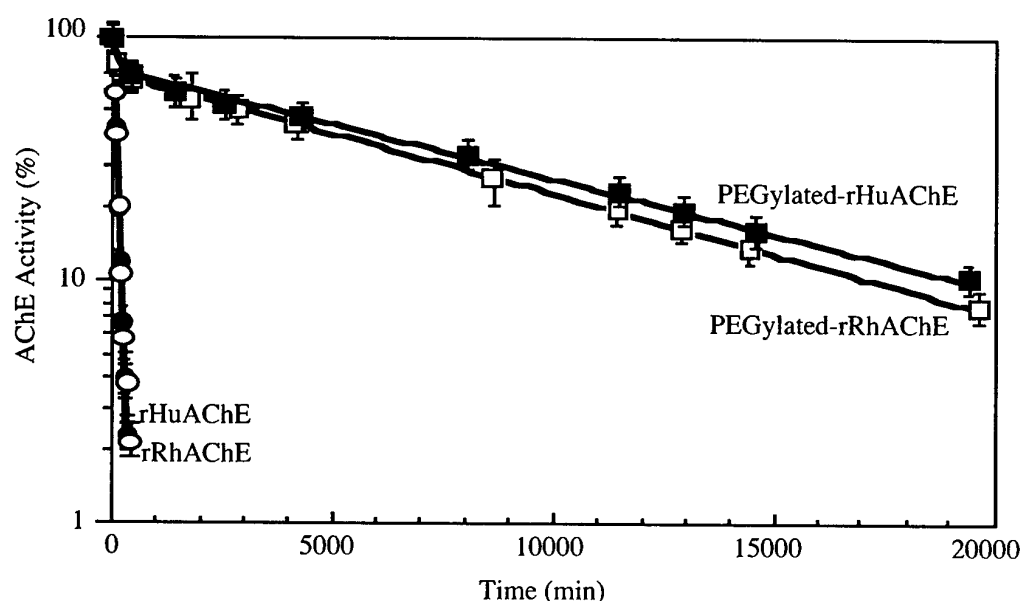
**The effect of masking of primate-specific amino-acid domains by PEGylation on the circulatory lifetime of AChE in rhesus macaques.**

Previous studies carried out in mice (Cohen *et al.*, 2001) demonstrated that the conjugation of PEG chains to lysine residues of AChE resulted in the generation of a molecule which resides in the circulation for exceedingly long periods of time. Most importantly, PEG conjugation prevented plasma clearance of undersialylated rHuAChE as well, suggesting that the appendage of PEG chains obstructs accessibility of domains at the surface of the enzyme to the hepatic asialoglycoprotein receptors. It was therefore interesting to examine whether PEGylation can prevent the putative species-specific amino-acid domain removal process, as well. To address this issue, partially sialylated rHuAChE and rRhAChE produced in HEK-293 cells and consisting mostly of dimeric forms were subjected to PEG appendage. To allow efficient PEG-conjugation of the two primate AChEs without compromising AChE bioactivity, the enzymes were reacted with succinimidyl propionate activated PEG at molar ratios and under conditions determined in the past (Cohen *et al.*, 2001). Under these conditions, the PEGylated product carries an average of 4 PEG chains per enzyme subunit and retains full enzymatic activity.



**Fig. 19: Mapping of divergent amino acids between BoAChE and RhAChE/HuAChE onto the three-dimensional model of HuAChE** The protein backbone is depicted as a line ribbon and the divergent residues are shown as space-fill spheres. Note that all divergent residues map to the enzyme surface. (A) Mapping of the BoAChE/RhAChE diverging amino acid residues within three enzyme surface-located domains. The BoAChE/RhAChE diverging amino acid residues are colored according to their location: red, Domain A; magenta, Domain B; and green, Domain C. Arrows point to the three divergent residues (Met42, His284 and Ala467) between RhAChE and HuAChE. (B) Mapping of the fourth glycosylation site (Asn 61) of BoAChE to the center of Domain A. (C) Mapping of the BoAChE/RhAChE divergent domains within the tetrameric structure of human AChE.

The PEGylated rHuAChE and rRhAChE were administered to rhesus macaques and the pharmacokinetic profiles of the chemically modified enzymes were monitored (Fig. 20). The PEGylated enzymes were retained in the circulation for exceedingly long periods of time, exhibiting MRT values of  $10,360 \pm 280$  min and  $9,900 \pm 390$  min for PEGylated rHuAChE and PEGylated rRhAChE, respectively. Thus, the chemical conjugation of PEG to the primate enzymes resulted in an extraordinarily high increase in the residence of the human and rhesus enzymes, over 100-fold, in the circulation of monkeys, demonstrating that PEGylation of AChE indeed leads to the efficient masking of the various determinants that promote enzyme clearance in monkeys. The finding that the PEGylated forms of the primate enzymes exhibit MRT values which are exceedingly higher than that of rBoAChE even in its fully processed form ( $1510 \pm 45$  min, See Table 5), demonstrates that the appendage of PEG chains masks not only the elements which cause the minor inequality in the pharmacokinetics of human and rhesus AChEs, but also overcomes the contribution of the primate-specific amino acid sequences to the inferior pharmacokinetic performance in monkeys of both rHuAChE and rRhAChE as compared to the bovine enzyme. In fact, the MRT values of the PEGylated primate AChEs are similar to that exhibited by native long-lived rhesus BChE (Fig. 18), indicating that PEG appendage results in the generation of enzyme forms which are no longer accessible to circulatory removal systems which recognize and interact with the self primate acetylcholinesterase-specific domains.



**Fig. 20: Pharmacokinetic profiles of PEGylated rRhAChE and PEGylated rHuAChE in rhesus macaques** Purified recombinant rhesus (empty symbols) or human (filled symbols) AChE generated in HEK-293 cells (partially sialylated, dimeric forms), were administered to 3 rhesus macaques before (circles) or after (squares) chemical conjugation to polyethylene glycol. The residual AChE activity in the circulation was assayed at the indicated time points as described in the "Experimental" section

In conclusion, the present study defines amino acid-related domains as an important element, which together with post-translation related factors such as oligosaccharide sialylation, enzyme assembly and glycan loading, determines the circulatory lifetime of acetylcholinesterase in the circulation of non-human primates. In fact, in the rhesus macaque animal system, amino-acid-related elimination of AChE is effective to a degree that it prevents full manifestation of the positive effect of efficient sialylation on the circulatory retention of the enzyme. Even so, chemical conjugation of PEG to AChE species containing amino acid epitopes which promote circulatory elimination, could effectively protect these enzymes from amino acid-related removal. The amino acid configurations that promote enzyme clearance in rhesus macaques seem to be restricted to those presented by "self" or "self-like" AChE species. Unlike in rhesus macaques, differences in amino-acid sequences had only a minor effect on the differential clearance of bovine, human and rhesus AChE in the mouse animal system yet this apparent insensitivity to amino acid alterations may stem from the fact that the three AChE species examined, BoAChE, HuAChE and RhAChE, are equally "foreign" and therefore do not present amino-acid epitopes which promote enzyme removal in mice. Indeed, primates are characterized by a paucity of soluble AChE in the circulation at the adult stage (Li *et al.*, 2000). The predominance of butyryl ChE in the circulation of adults, and the finding that "self" acetyl ChE is eliminated from the circulation, may suggest that organisms have evolved elaborate mechanisms to support circulatory residence of butyryl cholinesterase in particular. Although the biological significance of active elimination of soluble acetylcholinesterases from the bloodstream remains to be elucidated, it may be speculated that this process has to do with one or more of the non-catalytic functions attributed to this enzyme, which include cell differentiation, haematopoietic and thrombopoietic activities and involvement in cell-adhesion (Balasubramanian and Bhanumathy, 1993; Grisaru *et al.*, 1999; Soreq and Seidman, 2001).

## V. KEY RESEARCH ACCOMPLISHMENTS

1. Demonstration that PEGylation of AChE can overcome a quantitative deficiency in oligosaccharides side-chains, such as manifested by the monoglycosylated N350Q/N464Q rHuAChE mutant, resulting in the generation of circulatory long-lived molecules.
2. Demonstration that PEGylation of AChE can overcome inefficient glycan sialylation. Upon PEGylation, partially sialylated or totally asialylated HuAChEs manifest long-term circulatory residence.
3. Demonstration that PEGylation of AChE can overcome inefficient assembly into tetramers. Upon PEGylation, dimeric or monomeric HuAChEs manifest long-term circulatory residence.
4. Determination of the ability to extend by PEG-conjugation, the circulatory lifetime of AChE species exhibiting high-mannose type N-glycans or which are totally devoid of N-glycans.
5. The key findings, described in 1 to 4 above, suggest that large-scale production of recombinant human AChE in cost-effective production systems such as bacteria or yeast, could provide the means for generating circulatory long-lived PEGylated rHuAChE for therapeutic use.
6. Planning and construction of a human *ache* gene with altered codons (lower GC contents), optimized for expression of authentic human AChE, in microorganisms (e.g. *E. coli*, *Bacillus*, yeast).
7. Verification that the synthetic *ache* gene codes for a protein product specifically recognized by polyclonal anti-HuAChE antibodies.
8. Determination of the *Macaca mulatta* AChE sequence, generation of recombinant rhesus AChE (rRhAChE) in the HEK-293 cell system and its purification.
9. Determination of the pharmacokinetic profiles of asialylated, partially sialylated and fully sialylated rRhAChE in its non-tetramerized or tetramerized form, in mice. These studies allowed us to determine that circulatory retention of rhesus AChE in mice is governed by the same set of post-translation-related rules as the bovine and human enzyme forms.
10. Demonstration that primate AChEs (human and rhesus) are specifically removed from the circulation of rhesus macaques via species-specific amino-acid-related epitopes located at the



enzyme surface, in a manner which overrides the positive effect of enzyme subunit assembly and glycan loading on circulatory retention.

11. Demonstration that efficient conjugation of PEG to primate AChEs (human or rhesus) results in the generation of an enzyme species which resides in the circulation of rhesus macaques for very long periods of time (Mean residence time ~10,000 mins.).

12. Demonstration that native BuChE has a circulatory residence time comparable to that of PEG-AChE.

## VI. REPORTABLE OUTCOME

### List of publications related to the current contract

Shafferman, A., Barak, D., Kaplan, A., Ordentlich, N., Ariel, N. And Velan, B., (2003) The aromatic "trapping" of histidine 447 in catalysis of acetylcholinesterases. *VII International Meeting on Cholinesterases, Pucon Chile*. In press

Shafferman, A., Barak, D., Ordentlich, A., Kronman, C., Kaplan, D. and Velan B., (2003) Orchestration of the functional subsites in ache active center - contribution to catalytic perfection and reactivity toward specific ligands. *ADPD Conference, Seville*. Submitted.

Cohen, O., Kronman, C., Chitlaru, T., Velan, B. and Shafferman, A. (2003) Uses of chemically modified cholinesterases for detoxification of organophosphorus compounds. Patent application no. 10/476,338 applied to the U.S patent office by the state of Israel represented by IIBR.

Kaplan, D., Barak, D., Ordentlich, A., Kronman, C., Velan, B., and A. Shafferman (2004). Is aromaticity essential for trapping the catalytic His447 in human acetylcholinesterase. *Biochemistry*, in press.

Cohen, O., Kronman, C., Velan, B. and Shafferman, A. (2004). Amino-acid domains control the circulatory residence time of primate acetylcholinesterase in rhesus macaques. *Biochem. J.* 378, 117-128

Shafferman, A., Chitlaru, T., Ordentlich, A., Velan, B., and Kronman, C. (2004) A complex Array of Post-Translation Modifications Determines the Circulatory Longevity of AChE in a Hierarchical Manner. In: *Cholinergic Mechanisms*. In press.

Ordentlich, A., Barak, D., Ariel, N., Kronman, C., Kaplan, D., Velan, B. and Shafferman, A. (2004) Surprising findings from the functional analysis of Human AChE adducts of Alzheimer's disease drugs. In: *Cholinergic Mechanisms*. In press.

Cohen, O., Kronman, C., Chitlaru, T., Ordentlich, A., Velan, B. and Shafferman, A. (2004) Generation of pharmacokinetically improved recombinant Human Acetylcholinesterase by polyethylene glycol modification. In: *Cholinergic Mechanisms*. In press.

Kaplan, D., Ordentlich, A., Barak, D., Ariel, N., Kronman, C., Velan, B., and Shafferman, A. (2004) Attempts to engineer an enzyme-mimic of Butyrylcholinesterase by substitution of the six divergent aromatic amino acids in the active center of AChE. In: *Cholinergic Mechanisms*. In press.

Elhanany, E., Ordentlich, A., Dgany, O., Kaplan, D., Segall, Y., Barak, R., Velan, B., and Shafferman A. (2004) MALDI-TOF/MS analysis of tabun-AChE conjugate: A tool for resolution of "aging" pathway. In: *Cholinergic Mechanisms*. In press.

Kronman, C., Chitlaru, T., Seliger, N., Lazar, S., Lazar, A., Zilberstein, L., Velan, B., and Shafferman, A. (2004) Some basic rules governing oligosaccharide-dependent circulatory residence of glycoproteins are revealed by MALDI-TOF/MS mapping of the multiple N-glycans associated with recombinant bovine acetylcholinesterase. In: *Cholinergic Mechanisms*. In press.

Chitlaru, T., Kronman, C., Lazar, S., Seliger, N., Velan B. and Shafferman, A. (2004) Effect of post-translation modifications of human acetylcholinesterase on its circulatory residence. In: *Cholinergic Mechanisms*. In press.

## VII. CONCLUSIONS

Enhancement of the circulatory life span of recombinant AChEs is of crucial importance for their employment as therapeutic bioscavengers of OP compounds. Extensive studies involving characterization and modulation of recombinant AChE post-translation processing, firmly established that improved post-translation-related maturation contributes in a marked manner to their circulatory longevity. In parallel, a different set of experiments allowed us to determine that chemical modification of AChEs by conjugation of PEG molecules can also give rise to bioactive enzyme forms which are retained in the circulation for very long periods of time.

To define the possible interrelationship between PEG-conjugation and post-translation modifications of AChE enzyme forms with regard to circulatory residence, we examined the pharmacokinetic performance of well-defined AChE forms differing in their post-translation processing. This line of studies allowed us to determine that PEG-conjugation increased the circulatory retention of suboptimally processed AChE forms characterized by low glycan contents, inefficient sialylation or incomplete assembly into tetramers. Moreover, AChE species exhibiting altered glycan structures that do not conform with the classical complex-type of oligosaccharides typical of animal cell proteins or which were entirely devoid of glycan appendages, also displayed prolonged circulatory retention following PEG-conjugation. Most notably, the differently processed AChE forms displayed nearly equal circulatory residence time values subsequent to PEGylation. Taken together, these studies show that PEGylation of AChE overrides post-translation-related suboptimal processing and that the extent of circulatory retention of the chemically modified enzyme is determined solely by the appended PEG moieties.

During the course of the present research, we have documented a significant body of data regarding the factors contributing to AChE circulatory residence, all based on extensive studies carried out in the mouse animal model. We have now used the various AChE forms described above to profile their pharmacokinetic behavior in rhesus macaques (Cohen *et al.*, 2004). Although these pharmacokinetic studies were funded by sources other than USAMRMC, they have important implications to this contract. These studies in rhesus macaques allowed us to determine that: (1) Glycan sialylation, enzyme tetramerization and glycan loading determine circulatory retention of AChE in rhesus macaques, according to the hierarchical rules determined in the mouse animal system. (2) A mechanism for AChE removal, which was not previously detected in mice, facilitates AChE elimination from the circulation of rhesus macaques. (3) This mode of AChE removal is mediated through interactions with primate-specific AChE amino-acid epitopes, and seems to reflect a system which has evolved to eliminate "self" or "self-like" acetylcholinesterase in macaques. Thus, amino-acid-epitopes serve as an additional element, which together with post-translation-related factors, determine the circulatory fate of AChEs in non-human primates. (4) The controlled conjugation of PEG to primate (human or rhesus) AChEs, results in the generation of bioactive enzyme which resides

in the circulation of macaques for extraordinarily long periods of time, demonstrating that AChE shielding by PEG-appendage protects the enzyme from the amino-acid-related elimination system.

A key implication of these conclusions is that it is now possible to consider production of recombinant human AChE in low-cost microorganisms-based production systems. To this end, we commenced on the generation of a synthetic *ache* gene which has been adapted for efficient expression in microorganisms by lowering the GC contents of the coding sequences of the native human gene, and demonstrated that this synthetic gene expresses authentic AChE *in vitro*. Expression of this gene in *Bacillus* cells or in other microorganisms of choice, will allow us to evaluate which of these systems may serve in the future for the large-scale cost-effective production of this OP-bioscavenger.

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